Turning On Uracil-DNA Glycosylase Using a Pyrene Nucleotide Switch*

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Base flipping is a highly conserved process by which enzymes swivel an entire nucleotide from the DNA base stack into their active site pockets. Uracil DNA glycosylase (UDG) is a paradigm enzyme that uses a base flipping mechanism to catalyze the hydrolysis of the N-glycosidic bond of 2'-deoxyuridine (2'-dUrd) in DNA as the first step in uracil base excision repair. Flipping of 2'-dUrd by UDG has been proposed to follow a “pushing” mechanism in which a completely conserved leucine side chain (Leu-191) is inserted into the DNA minor groove to expel the uracil. Here we report a novel implementation of the “chemical rescue” approach to show that the weak binding affinity and low catalytic activity of L191A or L191G can be completely or partially restored by substitution of a pyrene (Y) nucleotide wedge on the DNA strand opposite to the uracil base (U/A to U/Y). These results indicate that pyrene acts both as a wedge to push the uracil from the base stack in the free DNA and as a “plug” to hinder its reinsertion after base flipping. Pyrene rescue should serve as a useful and novel tool to diagnose the functional roles of other amino acid side chains involved in base flipping.

A remarkable and evolutionarily conserved aspect of enzymatic recognition of damaged bases in DNA is the process of base flipping (1). This enzyme-induced conformational change in the DNA is a prerequisite for many enzymes to catalyze various chemical transformations on the base that require access to its functional groups. DNA glycosylases, which catalyze the first step in DNA base excision repair, are one general enzyme class that must act through a base flipping mechanism (2).

The most prevalent type of spontaneous DNA damage is that brought about by cytosine deamination, or the misincorporation of dUTP into DNA during replication, resulting in the presence of uracil in DNA. The cytosine deamination route leads to G → U mismatches that ultimately can lead to G → C to A → T transition mutations after two rounds of DNA replication. Thus, a uracil DNA glycosylase activity has evolved to combat this unrelenting source of genomic instability. Flipping of 2'-dUrd by UDG has been proposed to follow a “pinch-push-pull” mechanism in which a trio of serine residues pincches the DNA backbone producing a localized stress in the DNA (3–5), a completely conserved leucine residue (Leu-191) pushes through the minor groove to expel the uracil from the major groove (see Fig. 1A), and several enzyme hydrogen bond donors and acceptors pull and stabilize the extrahelical uracil in the active site. The pinch-push-pull mechanism appears to represent a highly conserved mechanism to promote base flipping, because corresponding interactions have been found in the structures of all DNA glycosylase-DNA complexes (6, 7).

The kinetic mechanism of base flipping has been studied in significant detail for Escherichia coli UDG (8). In stopped-flow fluorescence studies, the overall base flipping process has been shown to be extremely rapid (k_{flip} ~ 1200 s^{-1}), assisted by the enzyme, and about 10-fold faster than the chemical step of glycosidic bond cleavage (k_{cl} ~ 150 s^{-1}). The specificity of the enzyme for flipping and cleavage of uracil, as opposed to all other naturally occurring DNA bases, was shown to be derived from steric exclusion of other bases and from hydrogen bond complementarity of the active site with uracil (9). Consistent with these observations, UDG does not appear to be highly processive (10, 11), which argues against a scanning mechanism involving transient flipping of normal DNA bases (12).

Although the structural biology of DNA glycosylase-mediated base flipping has seen tremendous advances in the last few years (2), our understanding of the forces that give rise to extrahelical bases and the nature of the reaction pathway for base flipping is lacking. One approach to test our understanding of this process is to generate substrate analogs or enzyme mutants that lack functional groups that are hypothesized to be essential for the process, and then probe the damaging effect of the perturbation using biophysical methods. Conversely, the generation of substrate analogs or small molecules that “rescue” the damaging effects of enzyme mutations can provide valuable insights into the role of a functional group in the mechanism. In this report we use both approaches to test the proposed pushing role of Leu-191 of UDG in uracil flipping (see Fig. 1A). Deletion of this side chain results in a 10- to 625-fold decrease in k_{cl}/k_{m} using duplex DNA substrates containing a single U/P or U/A base pair (where P is the fluorescent adenine analog 2-aminopurine). We then tested the proposed role of this residue by inserting a pyrene nucleotide analog opposite to the uracil (Fig. 1A). We surmised that the bulky pyrene “base,” which fills the entire space normally occupied by the normal U/A or U/P base pair (see Fig. 1B), might serve as a mechanical wedge to either force the uracil from the DNA base stack in the

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† The abbreviations used are: UDG, uracil DNA glycosylase; wt, wild-type; P, 2-aminopurine (2-AP); HPLC, high performance liquid chromatography; U\textsuperscript{Y}, 2'-β-fluoro-2'-deoxyuridine nucleotide; ss, single-stranded; 2'-dUrd, 2'-deoxyuridine.
free DNA (pushing), or hinder its reinsertion once it is expelled in the UDG complex (plugging). We show here that pyrene completely rescues the damaging effects of the L191A and L191G mutations on site-specific DNA binding. In addition, pyrene totally rescues the damaging kinetic effects of the L191A mutation and partially rescues the kinetic parameters of L191G. The data support a mechanism in which pyrene wedges the uracil from the base stack in the free DNA, and then plugs the hole after the base departs (see Fig. 1C). One major role of Leu-191 appears to involve plugging the cavity that is left behind after the pinching forces have expelled the uracil, thereby increasing the lifetime of the extrahelical base. Because similar bulky amino acid side chains are involved in other DNA glycosylase base flips, a revised “pinch-push-plug-pull” mechanism for base flipping is suggested. These results further demonstrate the remarkable utility of nonpolar nucleoside analogs in the study of nucleic acid-protein recognition (13).

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis—The substrates and substrate mimics were synthesized using standard phosphoramidite chemistry with an Applied Biosystems 390 synthesizer. The nucleoside phosphoramidites were purified by anion exchange HPLC and the pyrene phosphoramidite was established by proton NMR and electrospray ionization-mass spectrometry. After synthesis and deprotection, the oligonucleotides were purified by anion exchange HPLC and desalted by C-18 reversed phase HPLC (Phenomenex Aqua column). The size, purity, and nucleotide composition of the DNA was assessed by analytical reversed phase HPLC, matrix-assisted laser desorption mass spectrometry, and denaturing polyacrylamide gel electrophoresis. The DNA strands were hybridized as previously described to form the duplexes used in the kinetic and binding studies as shown in Table I (8). In these sequences, P = 2-aminopurine deoxynucleotide, U" = 2'-fluoro-2'-deoxyuridine nucleotide, and Y = pyrene deoxyribonucleotide. The concentrations of the oligonucleotides were determined by UV absorption measurements at 260 nm, using the pair-wise extinction coefficients for the constituent nucleotides (16) and the measured extinction coefficient of 9.6 mm⁻¹ cm⁻¹ (260 nm) for the pyrene nucleoside in 40% methanol.

Purification of UDG—As previously described, UDG from E. coli strain B was purified to >99% homogeneity using a T7 polymerase-based overexpression system (9, 17). The concentration of the enzyme was determined using an extinction coefficient of 38.511 mm⁻¹ cm⁻¹ (18). The L191A and H187G mutants were generated using the QuickChange double-stranded mutagenesis kit from Stratagene (La Jolla, CA), and the mutations were confirmed by sequencing both strands of the DNA. The 6His-tagged mutant proteins were purified using nickel chelate chromatography as previously described (5). The His tag was removed by cleavage using biotinylated thrombin followed by purification using streptavidin beads and nickel chelate chromatography.

Thermal Denaturation Studies—Solutions of 3 μM of each DNA duplex were melted in Teflon-stoppered 1-cm path length quartz cells on a Varian Cary UV-visible spectrophotometer equipped with a thermoprogrammer. Absorbance was monitored at 260 nm, and the temperature was ramped from 5 to 80 °C at a rate of 0.5 °C/min. In all cases, the duplexes displayed a sharp and apparently two-state transition. Melting temperatures were determined from first derivative fits of absorbance with respect to 1/T.

KMnO₄ Sensitivity Measurements—Oxidative modification with KMnO₄ was performed with 100 mM 5'-29P-labeled 11mer duplex, in 20 μl of TMN buffer (10 mM Tris-HCl (pH 8.0), 2.5 mM MgCl₂, 25 mM NaCl). Reactions were initiated by the addition of KMnO₄ to a final concentration of 1 mM and incubated for 1 or 3 min at 15 °C. The reactions were quenched with 20 μl of a solution consisting of 1.5 M sodium acetate, pH 7.0, 1 M β-mercaptoethanol, and 50 μg/ml tRNA. The DNA was then precipitated by the addition of 3 volumes of ice-cold 100% ethanol. Modification-specific strand cleavage was performed by the addition of 100 μl of 1 M piperidine to the DNA pellet and heating at 90 °C for 20 min. The DNA samples were then ethanol-precipitated, washed twice with 80% ethanol, combined with 6 μl of denaturing load buffer (80% formamide, 1× TBE, 0.02% bromphenol blue, 0.02% xylene cyanol), and resolved on a 20% sequencing gel (65 watts of constant power for 1 h).

Steady-state Kinetic Measurements—The steady-state kinetics of uracil glycosylase bond cleavage were determined at 25 °C in TMN buffer using a steady-state fluorescence assay (19) or an analogous assay in which the increase in pyrene fluorescence due to glycosidic bond cleavage is followed. The steady-state kinetic parameters (kcat and kcat/KM) were obtained from plots of the observed rate constants (kobs) against substrate concentration ([S]tot) using a standard hyperbolic kinetic expression and the program Graft 5 according to eqs. 1 and 2.

\[
\text{h}_{\text{obs}} = \left(\frac{1}{\text{v}}\right)\text{K}_{\text{cat}}[\text{S}] + \text{[I]}
\]

In eq. 1, Kcat [S] is the initial rate in fluorescence units s⁻¹, ΔF = Kcat [S]/[I]tot, where ΔFtot is the total fluorescence increase for 100% conversion of a given substrate concentration ([S]tot) to product, and [UDG]tot is the total UDG concentration. The values for ΔF were determined by either letting the reaction go to completion, or by adding 10–20 nM wild-type UDG to rapidly bring the reaction to its endpoint after completing the initial rate measurements. For the time-based scans with 2-AP, an excitation wavelength of 310 or 320 nm was used, and the emission was observed at 370 nm. For pyrene-based measurements, an excitation wavelength of 350 nm was used, and the emission was observed at 580 nm. The reliability of the new pyrene fluorescence assay was validated by direct measurements of the time dependence of uracil release using an HPLC-based method (20).

Binding and Inhibition Studies—The dissociation constants (Kd) for binding of UDG to the DNA molecules indicated in Table I were determined using two methods. First, for the 2-aminopurine (2-AP) and pyrene-labeled molecules, direct fluorescence binding measurements were made by titrating fixed concentrations of the DNA with increasing amounts of UDG. To minimize background fluorescence from tryptophan residues of the enzyme, an excitation wavelength of 320 nm was used when changes in 2-AP fluorescence were followed, and 2-AP emission spectra in the range 340–450 nm were collected. The binding data were fitted to eq. 3 after the background fluorescence of UDG was subtracted from each spectrum (Fb and Ff are the initial and final fluorescence intensities, respectively). When pyrene fluorescence was followed, excitation was at 350 nm and emission spectra from 370 to 450 nm were collected. The fluorescence intensity (F) at 380 nm was plotted against [UDG]tot to obtain the Kd from eqs. 3 and 4.

\[
F = F_f - (F_f - F_b)\frac{[\text{DNA}]_{\text{tot}}}{2}b - (b - 4)\frac{[\text{DNA}]_{\text{tot}}}{[\text{DNA}]_i}F_f
\]

For the nonfluorescent DNA molecules, competitive kinetic inhibition studies were performed to obtain the Kd values. In most cases, conditions were chosen such that [UDG]tot was comparable to [I], and use of eq. 6 was required to calculate the Kd using the 2-aminopurine continuous fluorescence assay (19) or an analogous assay. When pyrene fluorescence was followed, excitation was at 350 nm and emission spectra from 370 to 450 nm were collected. The fluorescence intensity (F) at 380 nm was plotted against [UDG]tot to obtain the Kd from eqs. 3 and 4.

\[
F = F_f - [(F_f - F_b)\frac{[\text{DNA}]_i}{2}b - (b - 4)\frac{[\text{DNA}]_i}{[\text{DNA}]_i}F_f]^{\frac{1}{2}}
\]

In the case of D64N, where binding of the inhibitor was tight and [UDG]tot was comparable to [I], the use of eq. 6 was required to calculate [I] in eq. 5, using the conservation of mass equation [I] = [I]tot - [IE], where [IE] is the concentration of the enzyme-inhibitor complex, and [I] is the observed rate constant (v/[UDG]tot) at a given [I], and Kd is the observed rate constant in the absence of inhibitor, and

\[
h/K_I = 1/(1 + I/K_I)
\]

For the inhibition studies, a sensitive HPLC kinetic assay for monitoring the formation of the abasic product was employed using the previously characterized trinucleotide substrate ApUpAp (kcat = 23 ± 3 μM) (20).

Molecular Modeling—A model for the pyrene nucleotide in the context of a duplex DNA bound to L191A UDG was generated from the crystal coordinates of the ternary complex of human UDG bound to the products uracil and abasic DNA (Protein Data Bank entry 1SSP). A truncated model was used that included the flipped-out abasic nucleotide, the two flanking DNA base pairs, uracil and Leu-191 (Fig. 1A). The corresponding leucine side chain was mutated computationally to ala-
RESULTS AND DISCUSSION

Design and Characterization of Normal and Pyrene Wedge Substrates—Nonpolar nucleoside analogs that lack the hydrogen bond donor-acceptor groups of the natural DNA bases, yet mimic the shape of natural bases or even base pairs, have attracted much interest as probes of proteins-nucleic acid recognition (21). Most notably, the pioneering work of Kool and colleagues (22) has shown that DNA polymerase will specifically incorporate a pyrene (Y) nucleoside triphosphate (dYTP) opposite to DNA sites that lack bases, confirming that steric complementarity is an important component of high fidelity DNA replication. Although no high resolution structures of pyrene-containing DNA are yet available, thermal melting experiments and circular dichroism measurements indicate that pairing of pyrene against an abasic site, or even a natural base, is only modestly destabilizing and retains the B-form of the duplex (23).

On the basis that pyrene occupies the entire volume normally occupied by an entire DNA base pair, we envisioned that placing Y opposite to U might substitute for the role of Leu-191 in the process of uracil flipping by uracil DNA glycosylase (UDG). Our approach was to design duplex substrates for UDG in which Y was substituted for A or P opposite to U. We then tested whether the U/Y substrate selectively rescued the impaired binding and base-flipping activity of the L191A and L191G enzymes as compared with substrates with U/A or U/P base pairs. In addition, the pyrene rescue hypothesis predicts that other mutations of UDG that do not affect the base-flipping step would not be rescued by pyrene substitution. Two such mutations that have been previously investigated by our group are D64N, which removes the water-activating group, and H187G, which removes the catalytic electrophile (17, 24, 25). Thus, these mutant enzymes were also tested with the pyrene substrates.

The DNA sequences used in this study are shown in Table I. In addition to the insertion of the Y nucleotide, these sequences were designed to facilitate the kinetic and binding measurements. The incorporation of the fluorescent base 2-aminopurine (P) both opposite and adjacent to the excised uracil (A/P) allows continuous rate measurements of glycosidic bond cleavage (19). Both substrates were investigated to assess if kinetic differences exist between substrates with U/A versus U/P base pairs. In addition, the incorporation of the nonreactive deoxyuridine analog, 2'-fluoro-2'-deoxyuridine (U') allows binding measurements in the absence of bond cleavage (P/U'/AT/T, P/U'/AT/T) (8). All of the DNA sequences in Table I were shown to be entirely in the duplex form as judged by electrophoresis using a 19% native polyacrylamide gel with visualization by UV shadowing (not shown). The \( T_m \) values are found to be similar, falling in the range of 42.8–48.8 °C, which is about 18–24 °C higher than the temperature used for the kinetic and binding measurements (25 °C). These results confirm the previous observation that pyrene incorporation opposite to normal bases does not significantly disrupt the overall duplex stability (23).

**Table I**

| DNA       | Sequence          |
|-----------|-------------------|
| AU/A/TPT  | GCGCAUAGTGC       |
| PUA/TAT   | GCGCPUAGTGC       |
| AU'ATAT   | GCGCAU'AGTGC      |
| AU/TTY    | GCGCAUAGTGC       |
| PU/A/TAT  | GCGCPU'AGTGC      |
| AU'/TTY   | GCGCAU'AGTGC      |
| ssPUA     | GCGCPUAGTGC       |

**Fig. 1. Structural models of the flipped-out uracil bound to UDG.** A, crystallographic model of UDG bound to a U/A pair (4). B, structure of the pyrene base pair analog as compared with an adenine-thymidine pair. C, computational model for the complex of L191A UDG with substrate DNA containing a Y:U pair. The views in A and C are through the major groove of the DNA, and the hydrogens on the methyl groups of Leu-191 and Ala-191 are shown. The crystallographic model in C was derived from the crystallographic model as described under “Experimental Procedures.”
leading to cleavage of the glycosidic bond in the presence of piperidine, has long been used in DNA sequencing strategies (26), and as a probe of the solvent accessibility of T in single-stranded and duplex DNA (27). Here, we have used KMnO₄ sensitivity to address the question of whether Y pushes the opposite U or T from the DNA base stack, rendering it more accessible to oxidation. Indeed, the results shown in Fig. 2 clearly show that nucleotides U₆ and T₆ paired with A are relatively insensitive to KMnO₄ oxidation, but that U₆ and T₆ paired with Y show an enhanced sensitivity to oxidation. Careful quantification of the normalized intensities of the cleavage bands in Fig. 2 and two other experiments shows that U and T are 65 to 70% as sensitive to oxidation as the same bases in a single-stranded context (see legend to Fig. 2 for details). It should be clearly pointed out that the cleavage intensities of U₆ cannot be directly compared with T₆ in these experiments, because free uridine is about 5-fold less reactive than free thymidine to oxidation by KMnO₄ (28). Therefore, the sensitivities of U₆ or T₆ in the duplex context must be compared with the corresponding bases in the single-stranded form as we have done in Fig. 2B. It is also important to note that the enhanced sensitivity to oxidation cannot be explained by pyrene-induced duplex denaturation, because the T₉ base that is paired with A shows the same sensitivity regardless of whether there is a U₆(T₉)/A or U₆(T₉)/Y pair at position six (Fig. 2B). We conclude that the placement of pyrene opposite to U or T significantly increases the solvent accessibility of these bases and may preorganize these bases into an extrahelical conformation.

Fig. 2. Oxidation sensitivity of uracil and thymidine in the context of adenine and pyrene base pairs. A, samples of 5'-³²P-labeled single-stranded or duplex DNA with U/A, U/Y, T/A, or T/Y base pairs were incubated with 1 mM KMnO₄ for one or 3 min prior to treatment with piperidine. The DNA samples were subjected to electrophoresis on a 20% denaturing polyacrylamide gel, and the radioactivity in the gel was imaged and quantified. The sensitivity of the U and T bases to oxidation is revealed by the intensity of the cleavage bands in Fig. 2 and two other experiments shows that U and T are 65 to 70% as sensitive to oxidation as the same bases in a single-stranded context (see legend to Fig. 2 for details). It is also important to note that the enhanced sensitivity to oxidation cannot be explained by pyrene-induced duplex denaturation, because the T₉ base that is paired with A shows the same sensitivity regardless of whether there is a U₆(T₉)/A or U₆(T₉)/Y pair at position six (Fig. 2B). We conclude that the placement of pyrene opposite to U or T significantly increases the solvent accessibility of these bases and may preorganize these bases into an extrahelical conformation.

Catalytic Activity and DNA Binding of wtUDG with U/A, U/P, and U/Y Duplexes—The steady-state and single-turnover kinetic activity of wtUDG has been extensively studied in this laboratory using a variety of duplex and single-stranded substrate DNA molecules (5, 17). This work has led to the conclusion that the overall rate for UDG under K_cat conditions is severely limited by the product release step. This complicates the rigorous interpretation of the damaging effects of mutations derived from K_cat measurements, because the mutated enzymes are invariably rate-limited by the chemical step, rather than product release, leading to a large underestimate of the effects of the mutations. Fortunately, K_cat/K_m does not suffer the same shortcoming, because the product release step is not involved in this kinetic parameter (29), and comparisons between K_cat/K_m values for wild-type and mutant UDG enzymes generally provide quite good estimates of the true damaging effect of the mutations (20). Important for interpreting the studies here, the same complications hold true when comparing the K_cat values for substrates that have different product release rates. Thus, the most meaningful kinetic parameters for comparison under these conditions are K_cat/K_m and K_m.

Representative steady-state kinetic data for excision of uracil from various DNA substrates by wtUDG, L191A, and L191G. A, the reaction of wtUDG with AUA/TPT and AUA/TYT are shown along with the reaction for L191A with AUA/TPT and AUA/TYT. B, kinetic results for L191G with the UY and U/A substrates. The curves are the best fits of the data to eq. 2, and the kinetic results are summarized in Table II.
faster dissociation from the product complex (8, 17). The same
represented average values from multiple determinations. The reported errors are standard uncertainties.
pyrene enhances substrates containing U/A, U/P, and U/Y pairs, shows that T being 2.6-fold weaker than for A
k
120 s
-1

bias

results for wtUDG is that the placement of pyrene opposite to the kinetic measurements, pyrene substitution is found to have a small beneficial effect on the binding affinity of wtUDG to chemical step corresponding to Leu-191 of wtUDG is completely conserved in UDG sequences from bacteria to humans, suggesting a strategic role for this side chain in the cellular function of UDG. Despite its conservation and obvious key structural role

| Enzyme Substrate or mimic | K_d (μM) | k_cat (s^-1) | K_m (μM) | k_cat/K_m (s^-1) | MEa |
|---------------------------|----------|--------------|----------|------------------|-----|
| wtUDG sspUA               | 12.9 ± 0.7 | 0.42 ± 0.06  | 31 ± 5   |                  |     |
| AUA/TPT                   | 2.3 ± 0.2  | 0.065 ± 0.022| 35 ± 12  |                  |     |
| PUA/TAT                   | 5.3 ± 0.4  | 0.15 ± 0.03  | 36 ± 8   |                  |     |
| AUA/TYT                   | 19 ± 2     | 0.14 ± 0.04  | 135 ± 41 |                  |     |
| PU/A/TAT                  | 0.13 ± 0.05|              |          |                  |     |
| AU/P/A/TAT                | 0.063 ± 0.01|             |          |                  |     |
| AU/P/A/TYT                | 0.017 ± 0.002|            |          |                  |     |
| L191A                     | 3.4 ± 0.2  | 1.6 ± 0.3    | 2.1 ± 0.4| 0.067            |     |
| AUA/TPT                   | 2.9 ± 0.1  | 0.69 ± 0.10  | 4.2 ± 0.6| 0.12             |     |
| PUA/TAT                   | 0.64 ± 0.04| 1.0 ± 0.2    | 0.64 ± 0.12| 0.018         |     |
| AUA/TYT                   | 14 ± 1     | 0.11 ± 0.02  | 125 ± 24 | 0.93             |     |
| PU/A/TAT                  | 0.85 ± 0.13|            |          | 6.4              |     |
| AU/P/A/TAT                | 0.88 ± 0.09|            |          | 14               |     |
| AU/P/A/TYT                | 0.030 ± 0.005|           |          | 1.8              |     |
| L191G                     | 0.43 ± 0.01| 3.9 ± 0.03   | 0.11 ± 0.01| 0.0036         |     |
| AUA/TPT                   | 0.36 ± 0.02| 1.2 ± 0.2    | 0.29 ± 0.05| 0.008          |     |
| PUA/TAT                   | 0.05 ± 0.002| 0.89 ± 0.07| 0.007 ± 0.007| 0.0016    |     |
| AUA/TYT                   | 0.54 ± 0.04| 0.12 ± 0.02  | 4.6 ± 0.8 | 0.034            |     |
| PU/A/TAT                  | 5.1 ± 1.5  |            |          | 39               |     |
| AU/P/A/TAT                | 2.7 ± 0.4  |            |          | 43               |     |
| AU/P/A/TYT                | 0.030 ± 0.002|          |          | 1.8              |     |
| D64N                      | 0.029 ± 0.001| 0.069 ± 0.01| 0.42 ± 0.06| 0.014          |     |
| AUA/TPT                   | 0.015 ± 0.001| 0.053 ± 0.014| 0.28 ± 0.07| 0.007          |     |
| PUA/TAT                   | 0.039 ± 0.002| 0.029 ± 0.007| 1.3 ± 0.3| 0.036            |     |
| AUA/TYT                   | 0.033 ± 0.002| 0.029 ± 0.011| 0.83 ± 0.23| 0.006          |     |
| PU/A/TAT                  | 0.02 ± 0.002|            |          | 0.15             |     |
| AU/P/A/TAT                | 0.003 ± 0.0005|          |          | 0.05             |     |
| AU/P/A/TYT                | 0.005 ± 0.001|            |          | 0.29             |     |

a The mutational effect (ME) on k_cat/K_m or K_d for a given substrate is defined as (k_cat/K_m)mut/(k_cat/K_m)wt or (K_d)mut/(K_d)wt. The K_d values represent average values from multiple determinations. The reported errors are standard uncertainties.

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Representative binding of wild-type UDG and L191A to substrate mimic DNA. **A.** binding of AU/P/A/TYT to wtUDG as determined using the competitive inhibition assay. The line is the best fit of the data to eq. 5. **B.** binding of AU/P/A/TYT to L191A as determined using the direct pyrene fluorescence binding assay. The line is the best fit of the data to eqs. 3 and 4 (K_d > 35 ± 5 nM).
in base flipping (Fig. 1A), the steady-state kinetic studies using L191A and L191G indicate that uracil can still be excised from both duplex and single-stranded DNA substrates without this functional group, although much less efficiently (Table II). The detrimental effect of removing the two methyl groups of Leu-191 on the specificity constant $k_{cat}/K_m$ is found to be both sequence- and DNA structure-dependent. The $k_{cat}/K_m$ values of L191A with the AUA/TPT and PUA/TAT duplex substrates are 8.3- and 56-fold less than wtUDG, whereas the activity with the single-stranded substrate ssPUA is reduced by 14.7-fold. The data also reveal that the L191G enzyme is even more catalytically damaged than L191A (Table II). The $k_{cat}/K_m$ for L191G is reduced by 125- to 625-fold depending on the substrate, resulting from both $k_{cat}$ and $K_m$ effects. The binding affinities of L191A and L191G for the substrate mimic AU$^\bullet$A/TAT are decreased by 14- and 43-fold, confirming the observed mutational effects on $K_m$. The greater catalytic activity of L191A as compared with L191G likely reflects the residual beneficial effect of the additional methyl group, as might be expected from the proposed steric role of the full leucine side chain. The detrimental effects on $k_{cat}/K_m$ observed here fall in the same range recently reported for the L191A and L191G mutations using different substrates (8- to 80-fold). However, in this previous study binding measurements and DNA sequence effects were not investigated (30).

We conclude that Leu-191 is important but not essential for base flipping, and that removal of this residue has a similar detrimental effect on the excision of uracil from single-stranded and duplex DNA. This observation requires that the effect of Leu-191 be realized at a step that is shared by both single-stranded and duplex DNA. This step likely follows the partial or complete expulsion of the uracil from the duplex, because significant differences would be expected in the action of L191A or L191G on single-stranded and duplex DNA substrates if this residue were involved in actively pushing the uracil from its nested position in the DNA base stack. This conclusion is further supported by the pyrene rescue results.

**Specific Rescue Using the Pyrene Wedge**—In substantial contrast with wtUDG, the specificity constants of L191A and L191G for the substrate containing the U/Y pair are increased by 16- to 195-fold as compared with the substrates containing the U/P and U/A pairs, respectively (Table III and Fig. 5). In fact, pyrene completely restores the damaging effect on each kinetic parameter resulting from the L191A mutation (Tables II and III) and produces a 28- to 170-fold increase in the binding affinity of L191A and L191G for the substrate mimic DNA. It is interesting that both L191A and L191G show 5- to 6-fold larger pyrene rescue effects for $k_{cat}/K_m$ when the reference substrate has a U/A base pair as opposed to a U/P base pair (Table III). A likely explanation for this difference is that the ~0.5 kcal/mol lower stability of the U/P base pair (as estimated from stability measurements of T/P pairs (31)) renders the uracil easier to flip as compared with the U/A pair. In control experiments, the substrate AU/TAT gave indistinguishable kinetic results from PUA/TAT, eliminating the possibility that the reactivity difference between AU/A/TPT and PUA/TAT arises from the substitution of P for A at the 5′ position (data not shown). The identical or very similar rescue effects for the substrates AU$^\bullet$A/TAT and PU$^\bullet$A/TAT further reinforce this kinetic control (Table III).

The rescue effect is specific for the Leu-191 deletion mutations, because the kinetic and binding parameters for D64N are essentially the same for the U/A and U/Y pairs (Table III). The D64N mutation, which removes the carboxylate group that activates the water nucleophile and increases the kinetic barrier of the chemical step by 3000-fold (17), has been previously shown to have only a modest effect on DNA or uracil binding. Thus, the null effect of pyrene substitution for this mutant provides a dramatic demonstration of the specificity of the pyrene rescue for mutations that affect the base-flipping step. A similar null pyrene effect was obtained with the H187G mutant (not shown), which forms a strong interaction with uracil O2 in the transition state, yet also has little effect on DNA binding or base flipping (17). We anticipate that pyrene rescue will be a useful tool to further elucidate the functional roles of other participants in base flipping such as the serine side chains involved in phosphodiester compression (pinching).

**A Conceptual Framework for Uracil Flipping**—In a previous rapid kinetic study of base flipping by UDG, we proposed that the enzyme paid the energetic cost for flipping the uracil base by deforming the duplex before the base-flipping step (8). This proposal was supported by the surprising observation that
Supporting the conclusion that a major role of Leu-191 is to impede the exit cause, if the mechanism only involved active expulsion of the uracil by UDG, suggesting that plugging may be a common component in the mechanism for stabilizing extrahelical bases (6, 7). Can UDG mutants that are defective in flipping be targeted to specific uracils or even other bases in DNA by pyrene anti-sense rescue? Although we have shown at most a 200-fold pyrene rescue effect on the specificity constant of L191A, it may be possible to increase selectivity for U/Y sites by making further mutations directed at the flipping step or perhaps by

duplexes containing U/A and U/G pairs, as well as single-stranded U DNA had similar internal equilibrium constants for base flipping ($K_{gau}$) and essentially identical rate constants for dockling the uracil into the active site pocket. This led to the conclusion that, during the initial encounter complex with the DNA, UDG used binding energy to destabilize the duplex such that extrusion of the uracil was facilitated. In the discussion of the current results, it is useful to divide the overall free energy change for base flipping ($\Delta G_{obsd}$) into two discrete free energy components, a destabilization term ($\Delta G_{D}$) that represents all unfavorable changes that are necessary to set up for the base flip, and a favorable term ($\Delta G_{S}$) that represents all the stabilizing interactions that are gained as the base is nestled into its final resting place in the active site (Fig. 6). Thus, with sufficient structural and functional information, it may be possible to classify mutational (or substrate) effects as contributing to destabilization ($\Delta G_{D}$) or stabilization ($\Delta G_{S}$). Interpretations using this simple framework are not without peril, because the only measurable parameter is the net effect $\Delta G_{obsd} = \Delta G_{D} + \Delta G_{S}$, and some mutations could act to both stabilize and destabilize. Nevertheless, this framework is useful in the current case and provides a qualitatively consistent view of the role of Leu-191 and pyrene in the process of stabilizing the extrahelical uracil.

Consideration of these results suggests distinct energetic roles for pyrene and Leu-191 in promoting productive binding of the extrahelical base. Because the removal of Leu-191 has a similar detrimental effect on the excision of uracil from single-stranded and duplex DNA, and the rates of base flipping for wtUDG are indistinguishable for duplex and single-stranded DNA (8), then Leu-191 is likely to act after the initial duplex destabilization step. This conclusion seems inescapable, because, if the mechanism only involved active expulsion of the uracil by Leu-191, then the detrimental effect of its removal would certainly be greater for duplex DNA than for single-stranded DNA. This suggests an additional role for Leu-191 as a block, or plug, to hinder reinsertion of the uracil into the duplex or single-strand stack.2 In support of a similar role for Leu-191 in enhancing the binding of single and double-stranded DNA, the recent crystal structure of E. coli UDG bound to UAAp shows that Leu-191 resides only 3.5 Å from the modeled position of the 3’-flanking deoxyribose in this complex, suggesting it could easily serve as a plug to impede exit of the single-stranded DNA from the active site (5). A similar position for Leu-191 is seen in the structures of duplex DNA bound to human UDG (3, 4).

Pyrene is likely to serve an analogous plugging role as Leu-191 but may also serve to diminish the penalty for duplex destabilization by preorganizing the U in an extrahelical position (upper pathway in Fig. 6).2 Such extrahelical preorganization by pyrene would be expected to enhance the binding of wtUDG to the U/Y duplex, which is indeed observed (Table II), although the magnitude of this effect is fairly small (~ 0.7 kcal/mol). The much larger effect of pyrene on the binding affinities of the L191A and L191G mutants, which are defective in positioning or holding the uracil in its productive extrahelical position, suggests that the major role of pyrene, like Leu-191, is to increase the lifetime of the extrahelical uracil by a plugging mechanism.

A model depicting the roles of Leu-191 and pyrene in promoting productive base flipping is shown in Fig. 6. We speculate that serine pinching is involved in destabilization of the duplex before the extrusion step ($\Delta G_{D}$), which leads to the stabilizing interactions of Leu-191 and the other active site groups that interact with the extrahelical uracil ($\Delta G_{S}$). Further support for these ideas will follow from rapid kinetic investigations of the various base flipping mutants of UDG.3

Implications—The crystal structures of three other glycosylase-DNA complexes have revealed that a bulky amino acid side chain consistently resides in the position occupied by Leu-191 of UDG, suggesting that plugging may be a common component in the mechanism for stabilizing extrahelical bases (6, 7). Can UDG mutants that are defective in flipping be targeted to specific uracils or even other bases in DNA by pyrene anti-sense rescue? Although we have shown at most a 200-fold pyrene rescue effect on the specificity constant of L191A, it may be possible to increase selectivity for U/Y sites by making further mutations directed at the flipping step or perhaps by

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2 If the major role of Leu-191 is to increase the lifetime of the extrahelical base by a plugging mechanism, then it would be expected that the L191A mutation would increase the dissociation rate of the productively bound DNA, with a lesser effect on the association rate. Detailed stopped-flow fluorescence studies on the binding of U/A and U/Y DNA to wtUDG, L191A and other base-flipping mutants will be reported elsewhere (Y. L. Jiang and J. T. Stivers, manuscript in preparation). However, the present data using L191A clearly demonstrate that this mutation decreases the off-rate of U/A DNA by over 7-fold, with only a 3-fold effect on the on-rate. In addition, pyrene enhances the DNA association rate for the L191A mutant by 5-fold and slows the dissociation rate by 3.5-fold as compared with U/A DNA. These results support the conclusion that a major role of Leu-191 is to impede the exit of the extrahelical base and confirm that pyrene rescue is composed of two effects: preorganization of the extrahelical uracil in the free DNA and plugging the hole to hinder reinsertion of uracil after its flipping into the active site.

3 Y. L. Jiang and J. T. Stivers, unpublished observations.
altering the solution conditions. Because active site mutants of UDG have already been shown to possess catalytic promiscuity by removing T or C bases (32), it may also be possible to target specific C/Y or T/Y sites in DNA using this pyrene rescue strategy. Given the enormous catalytic power of UDG, a large part of which resides in fairly nonspecific interactions with the DNA backbone (5, 20), UDG offers an exceptional scaffold for engineering new glycosylase activities.

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