Base-Flipping Mutations of Uracil DNA Glycosylase: Substrate Rescue Using a Pyrene Nucleotide Wedge†

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ABSTRACT: We recently introduced a new substrate rescue tool for investigating enzymatic base flipping by uracil DNA glycosylase (UDG) in which a bulky pyrene nucleotide wedge (Y) was placed opposite a uracil in duplex DNA (i.e., a U/Y pair), thereby preorganizing the target base in an extrahelical conformation [Jiang, Y. L., et al. (2001) *J. Biol. Chem. 276*, 42347−54]. The pyrene wedge completely rescued the large catalytic defects resulting from removal of the natural Leu191 wedge, presumably mimicking the pushing and plugging function of this group. Here we employ the pyrene rescue method in combination with transient kinetic approaches to assess the functional roles of six conserved enzymatic groups of UDG that have been implicated in the "pinch, push, plug, and pull" base-flipping mechanism (see the preceding paper in this issue). We find that a U/Y base pair increases the apparent second-order rate constant for damaged site recognition by L191G pushing mutation by 45-fold as compared to a U/A pair, thereby fully rescuing the kinetic effects of the mutation. Remarkably, the U/Y pair also allows L191G to proceed through the conformational docking step that is severely comprised with the normal U/A substrate, and allows the active site of UDG to clamp around the extrahelical base. Thus, pyrene also fulfills the plugging role of the Leu191 side chain. Preorganization of uracil in an extrahelical conformation by pyrene allows diffusion-controlled damage recognition by all of these base-flipping mutants, and allows the UDG conformational change to proceed as rapidly as the rate of uracil flipping with the natural U/A base pair. Thus, the pyrene wedge substrate allows UDG to recognize uracil by a lock-and-key mechanism, rather than the natural induced-fit mechanism. Unnatural pyrene base pairs may provide a general strategy to promote site-specific targeting of other enzymes that recognize extrahelical bases.

Although removal of an enzyme group by mutagenesis is a powerful approach for inferring its energetic role in the overall reaction, it is often difficult to confidently assign the functional role of a given group from kinetic or thermodynamic analysis of a mutagenic effect alone. An elegant approach to link the functional role of an amino acid side chain with the energetic consequence of its deletion is to use chemical rescue (*1*, *2*). In its simplest form, chemical rescue involves the partial or complete substitution of the function of the side chain by adding back a small molecule to the reaction that shares the chemical functionality of the deleted side chain. Thus, the damaging effect of the mutation is rescued by the exogenous small molecule, providing an orthogonal test of the side chain function. A conceptually similar approach is to use a reactive substrate analogue that does not require the interaction with the enzyme side chain to undergo the transformation to product. An example of this "substrate rescue" approach would be to substitute a poor leaving group of a substrate with an activated leaving group, thereby bypassing the need for enzyme-assisted activation, and rescuing the damaging effect of removing the enzyme side chain involved in leaving group activation (*3*−*5*). In the past, such approaches have only been applied to chemical functional groups such as putative general acids, bases, or active site nucleophiles.

We have recently introduced a new application of the substrate rescue approach to assist in understanding the function of amino acid side chains purported to be involved in the overall process of base flipping by uracil DNA glycosylase (UDG) (*6*). The approach involves the incorporation of a bulky pyrene nucleotide analogue opposite the uracil base in duplex DNA (Figure 1). This analogue was anticipated to fill the entire space normally occupied by the normal U/A base pair (*7*), thereby serving as a mechanical wedge to expel the uracil from the DNA base stack in the free DNA, or hinder its reinsertion after base flipping. Here we extend the usage of pyrene rescue to examine the functional roles of the groups involved in the pinch, push,

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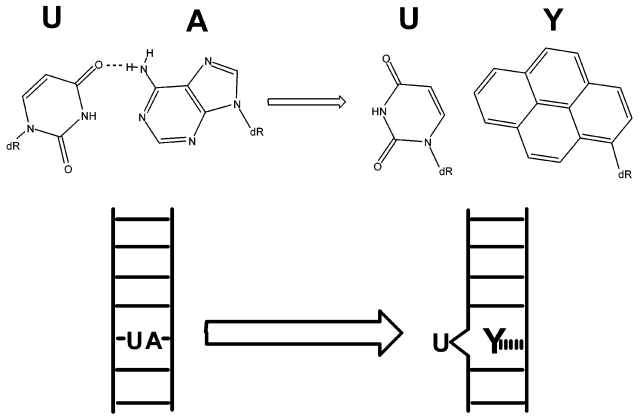

FIGURE 1: Structure of a U/A base pair as compared to a uracil−pyrene pair (U/Y). The large planar pyrene ring occupies about the same volume as the entire Watson−Crick base pair. Thus, incorporation of pyrene opposite uracil would be expected to act as a wedge to force the uracil from the base stack (bottom). This expectation has been previously established using potassium permanganate sensitivity experiments (*6*).

plug, and pull mechanism for base flipping. As described in the preceding paper in this issue (*8*), base flipping proceeds by a three-step mechanism. After nonspecific binding to the DNA, the uracil is destabilized and pushed into a metastable extrahelical state that is subsequently docked into the enzyme active site through a conformational clamping motion of the enzyme. In general, removal of the enzyme side chains that are directly involved in the base-flipping process retards the second-order rate constant for formation of the first metastable intermediate. In addition, the L191A, N123G, and S88A:S189A mutations severely destabilize the final docked conformation. Here we show that the pyrene substrate wedge allows wild-type UDG and all of these defective mutants to rapidly attain the final docked state. The wedge works by first allowing direct complexation of the enzyme with the extrahelical base in the free DNA by a lock-and-key mechanism, and then by accelerating the rate of the conformational change and enhancing the stability of the closed conformation. These results provide a novel example of how conformational preorganization of a substrate can lead to enhanced site-specific recognition by an enzyme.

EXPERIMENTAL PROCEDURES

Improved Synthesis of Pyrene Nucleoside Phosphoramidite. The β anomer of the pyrene (Y)[1] nucleoside phosphoramidite was synthesized in our laboratory by an improved procedure as described in the Supporting Information (*9*). The concentrations of the oligonucleotides were determined by UV absorption measurements at 260 nm, using the pairwise extinction coefficients for the constituent nucleotides, and the measured extinction coefficient of 9.6 mM^{-1} cm^{-1} (260 nm) for the pyrene nucleoside in 40% methanol.

Materials. The expression and purification of UDG and its mutants, and the purification and hybridization of the pyrene duplex (U^F/Y), is described in the previous paper in this issue (*8*). The sequence of this duplex is

5′ GCGCAU^{F}AGTCG 3′

3′ CGCGTY TCAGC 5′

DNA Binding and Kinetic Studies. The dissociation constants (K_D) for binding of the various enzymes to the U^F/Y DNA analogue were determined using three methods. All measurements were performed in TMN buffer, 10 mM Tris−HCl (pH 8.0), 2.5 mM $MgCl_2$, 25 mM NaCl, at 25 °C. In the first method, direct binding measurements were made by following the increase in pyrene fluorescence upon titrating fixed concentrations of the pyrene-containing DNA with increasing amounts of UDG. Excitation was at 350 nm, and emission spectra from 370 to 450 nm were collected using a Spex Fluoromax 3 spectrofluorimeter (*6*). The fluorescence intensity (F) at 380 nm was plotted against $[UDG]_{tot}$ to obtain the K_D from eq 1 in the preceding paper (*8*). In the second method, competitive kinetic inhibition measurements were performed using the substrate ApUpAp (*6*). Conditions were chosen whereby $[UDG]_{tot} \ll [U^F/Y]$ and [ApUpAp], and [ApUpAp] $\ll K_m$. Accordingly, K_i could be obtained directly from a plot of k/k_o against $[U^F/Y]$ as shown in eq 1,

$$k/k_o = 1/(1 + [U^F/Y]/K_i) \quad (1)$$

where k is the observed rate constant ($v/[UDG]_{tot}$) at a given $[U^F/Y]$ and k_o is the observed rate constant in the absence of the inhibitor. For these measurements, a sensitive HPLC kinetic assay for monitoring the formation of the abasic product was employed (*10*). Finally, dissociation constants were determined by following the tryptophan fluorescence decrease in UDG as U^F/Y DNA binds. For these experiments using the U^F/Y analogue, excitation was at 300 nm to minimize excitation of pyrene, and tryptophan emission scans were performed over the range 330−450 nm. Corrections for dilution and inner filter effects were made using the equation $F_{corr}(335\ nm) = F_{obsd} \times 10^{A^{300}/2}$, where A^{300} is the absorption of the DNA ligand at the excitation wavelength. F_{corr} was then plotted against $[DNA]_{tot}$ to obtain the K_D using eq 1 in the preceding paper (*8*).

The observed rate constants for association of the U^F/Y DNA with the various enzymes were obtained using an Applied Photophysics 720 stopped-flow fluorescence instrument (Surrey, U.K.) under pseudo-first-order conditions in which the concentration of the enzyme was always more than 4-fold greater than the concentration of the labeled DNA. The pyrene fluorescence increase as a function of time was recorded using a 360 nm cutoff filter with excitation at 333 nm. When tryptophan fluorescence was followed, excitation was at 300 nm and a 320 nm cutoff filter was used. The experiments were performed and analyzed as described in the preceding paper (see eqs 4−6) (*8*).

The dissociation rate constant (k_{off}) of U^F/Y from the various enzymes was also measured using irreversible conditions. When pyrene fluorescence was followed, these experiments were carried out by rapidly mixing a preformed U^F/Y−UDG complex with a large excess of nonfluorescent single-stranded trapping DNA. The sequence of the trap DNA was the same as that of the AU^{F}A strand of the duplex U^F/Y. The time-dependent decrease in pyrene fluorescence was then followed using the stopped-flow fluorescence

[1] Abbreviations: wtUDG, wild-type uracil DNA glycosylase; 2′-FU, 2′-fluoro-2′-deoxyuridine; 2-AP, 2-aminopurine; Y, pyrene nucleotide; U^F, 2′-fluoro-2′-deoxyuridine nucleotide in DNA.

Table 1: Equilibrium and Kinetic Constants for Binding of Wild-Type and Mutant UDG Enzymes to the U^F/Y DNA Analogue[a]

enzyme	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	k_{max} (s^{-1})	K' (μM^{-1})	k_{off}/k_{on} (μM)	K_D (μM)
wtUDG	520 ± 100	6.0 ± 0.2	1000 ± 170	0.52 ± 0.15	0.012 ± 0.01	0.011 ± 0.01
S88A	344 ± 8	63 ± 2			0.19 ± 0.01	0.12 ± 0.02
S189A	297 ± 6	28 ± 1			0.094 ± 0.004	0.083 ± 0.016
S88A:S189A	319 ± 4	300 ± 42			0.94 ± 0.13	1.5 ± 0.3
L191A	386 ± 15	22 ± 6			0.059 ± 0.016	0.030 ± 0.005
L191G	443 ± 86	34 ± 6			0.077 ± 0.020	0.030 ± 0.003
N123G	266 ± 51	402 ± 11			1.5 ± 0.3	1.9 ± 0.2
H187G	164 ± 3	4.8 ± 0.6			0.029 ± 0.004	0.034 ± 0.002
D64N	490 ± 8	6.0 ± 0.6			0.012 ± 0.002	0.005 ± 0.001

[a] For wtUDG, k_{on}, k_{off}, k_{max}, and K' were determined from fits to eq 6 of the preceding paper in this issue (*8*). For the mutants, k_{on} and k_{off} were obtained from linear regression fits to eq 5 of the preceding paper (*8*).

instrument. When tryptophan fluorescence was followed, the free enzyme was trapped using a high concentration of nonspecific DNA (50 μM). In all cases, the kinetic traces were fitted to a single-exponential equation (eq 7 of the preceding paper) (*8*).

RESULTS AND DISCUSSION

Definition of the Pyrene Rescue Effect. To allow easy comparison of the mutational effects with the pyrene rescue effects, we have defined the pyrene rescue as the kinetic (or binding) parameter for the U^F/Y analogue divided by the corresponding parameter for the U^F/A analogue (see the preceding paper in this issue) (*8*). Thus, pyrene rescue effects greater than unity always indicate the fold *enhancement* of a given parameter provided by the pyrene base pair as compared to the normal base pair.

Nature of Pyrene Rescue. An important prerequisite for interpreting a chemical or substrate rescue experiment is to understand the physical basis for the rescue effect. For traditional approaches that use small molecules or activated substrates to rescue chemical functionalities such as enzymatic general acids or bases, the interpretation is chemically intuitive and straightforward. However, in the case of pyrene, which acts as a mechanical wedge to promote the extrahelical state of uracil in duplex DNA, the interpretation requires more consideration.

In principle, pyrene could act in two general ways. First, pyrene might preorganize the uracil in a productive extrahelical state in the free DNA that is favorable to UDG binding. Since open base pairs exist at concentrations less than 1/1000 of the closed state at ambient temperature (*11*−*13*), increasing the concentration of an extrahelical conformation that is productive for enzyme binding would lead to an enhanced second-order rate constant for association of the enzyme with the substrate.[2] This pyrene effect would also lead to an increase in affinity (a decrease in K_D) that is directly proportional to the increase in the association rate (i.e., $K_D = k_{off}/k_{on}$). A hallmark for this mode of pyrene rescue is that it occurs in the free DNA, and would therefore be observed for both wild-type and mutant UDG enzymes. In support of this preorganization mechanism for pyrene rescue, we have shown that uracil or thymine in a U/Y or T/Y base pair is ~14-fold more sensitive to oxidation by permanganate as compared to the same bases in a U/A or T/A context, and that wtUDG shows 12-fold tighter binding to DNA containing a U/Y base pair as compared to a U/A base pair (see below). A second distinct mode for the pyrene rescue would be to enhance enzyme interactions with the bound extrahelical base, or a transition state leading to this extrahelical state. Accordingly, if removal of an enzyme side chain destabilizes a transition state(s) or ground state(s) in the overall process of base flipping, then pyrene might substitute for this role and rescue the mutational effect. We have previously reported an example of this effect with the L191A pushing mutation. This mutation decreases k_{cat}/K_m by 60-fold as compared to wtUDG, and its function can be completely replaced by the U/Y base pair (*6*). Measurement of the pyrene rescue effect on several kinetic or binding parameters allows assessment of the role of a side chain interaction at several discreet steps along the reaction pathway. In the studies described below, we have performed rescue experiments that probe the role of these eight mutations at multiple steps along the base-flipping pathway.

Pyrene Rescue of Equilibrium Binding Defects. We employed three different assays to measure the binding affinity of DNA containing a U^F/Y pair. The first assay was based on the increase in pyrene fluorescence upon U^F/Y binding to UDG, the second was a competitive kinetic inhibition assay (*6*), and the third was based on the decrease in the UDG tryptophan fluorescence upon binding uracil-containing DNA (*14*). We have previously reported a binding constant of 11 nM for wtUDG binding to the U^F/Y DNA construct using the kinetic inhibition assay (Table 1). Representative binding data using the pyrene fluorescence assay are shown in Figure 2A for the L191G mutant, from which a $K_D = 30 \pm 3$ nM was obtained. This K_D is 170-fold lower than for L191G binding to the U^F/A analogue, and is nearly the same as that for wtUDG, establishing that the pyrene wedge is an excellent surrogate for the naturally occurring leucine wedge. The majority of the other mutant enzymes show pyrene rescues on the binding equilibrium in the range 5−10, independent of the magnitude of the mutational effect. These rescue effects are similar to the 12-fold effect seen for the wild-type enzyme (Table 2). The comparable pyrene rescues for wtUDG and many of the mutations suggests that pyrene exerts this beneficial effect by preorganizing the uracil in the free DNA, and does not act by actively stabilizing the bound extrahelical state for these mutants that retain the natural Leu191 wedge. The

[2] NMR imino exchange rate measurements indicate that [out]/[in] for DNA base pairs is in the range 10^{-6} to 10^{-3}. We do not imply that the extrahelical state that is detected by imino exchange is necessarily a conformation that is productive for enzyme binding. However, these dynamic base pair breathing processes may provide seeding motions that are on the reaction coordinate for enzymatic base flipping (*10*, *11*).

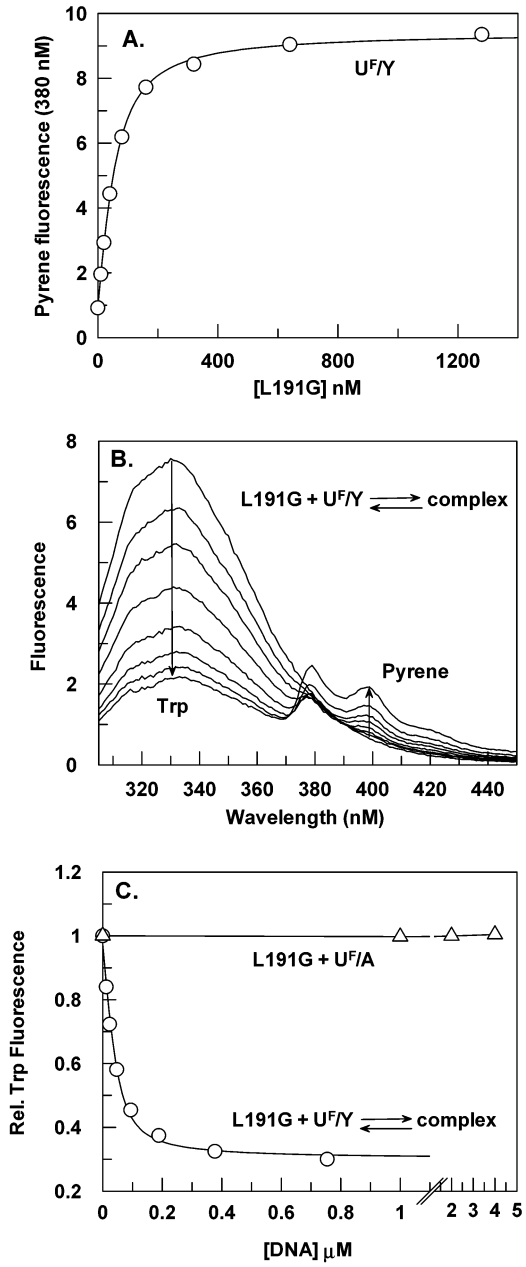

FIGURE 2: Specific DNA binding of L191G to U^F/Y. (A) U^F/Y DNA (50 nM) was titrated with increasing amounts of L191G, and the pyrene fluorescence increase at 380 nm is plotted as a function of [L191G]. The curve is the best fit to eq 1 in the preceding paper (*8*). (B) L191G (50 nM) was titrated with increasing amounts of U^F/Y DNA, and (C) the tryptophan fluorescence decrease at 335 nm is plotted as a function of [U^F/Y]. The K_D values are reported in Table 2. For comparison, the titration of L191G with the AU^F/A analogue is also shown.

larger rescue effects for the Leu191 deletion mutants, which are similar to the mutational effects, suggest that an additional mode of pyrene rescue becomes important when the leucine wedge is removed.

Pyrene Promotes Enzyme Docking around the Flipped Uracil. The large observed rescues of the Leu191 deletion mutants suggested that the U^F/Y analogue may have allowed these enzymes to achieve the closed UDG conformation that was not detected with the U^F/A analogue (see the preceding paper in this issue) (*8*). To test this possibility, we titrated L191G with the U^F/Y analogue and monitored the tryptophan emission spectrum (Figure 2B). As would be expected for the conformational docking step, the tryptophan fluorescence of L191G decreased in a hyperbolic fashion as the U^F/Y analogue bound, from which a $K_D = 10 \pm 3$ nM was obtained (Figure 2B,C). A similar tryptophan fluorescence decrease was also observed upon U^F/Y binding to N123G ($F_{bound}/F_{free} = 0.55$). Interestingly, although the conformational change is promoted by pyrene, facilitation of this conformation does not rescue the 16-fold binding defect of N123G (see Table 2 and also Table 4 of the preceding paper) (*8*). The serine double mutant showed only a small 20% tryptophan fluorescence decrease even when the DNA concentration was 4-fold greater than the K_D value for binding (not shown), suggesting that pyrene cannot fulfill the roles of these side chains in stabilizing the E*F complex. We conclude that pyrene provides a driving force that rescues the conformational step for the Leu191 and N123G mutants, but that this additional "push" does not lead to thermodynamically stable docking of the uracil base in the absence of the Asn123 side chain, or when both serine side chain interactions are deleted. Thus, the interactions of the pinch, push, plug, and pull groups are energetically coupled as revealed by these selective pyrene rescue effects.

Table 2: Comparison of Mutational and Pyrene Rescue Effects[a]

	mutational effects (UF/Y substrate)			pyrene rescue effects [(UF/Y)/(UF/A)]			
enzyme	$1/K_D$	k_{on}	$1/k_{off}$	$1/K_D$	k_{on}	$1/k_{off}$	k_{max}
wtUDG				11.8	3.2	3.5	1.5
S88A	10.9	1.5	10.5	10	7.3	1.3	
S189A	7.5	1.8	4.7	7.5	6.5	0.82	
S88A:S189A	88	1.6	50	5.6	23	0.68	
L191A	2.7	1.3	3.7	32	19	2.5	
L191G	2.7	1.2	5.7	170	45	2.7	
N123G	172	2.0	67	1.1			
H187G	3.1	3.2	0.8	8.2	4.8	0.96	
D64N	0.45	1.1	1.0	4.0	2.3	1.5	

[a] Mutational effects greater than unity indicate a damaging effect, in other words a decrease in the on-rate or an increase in the off-rate. The mutational effects using the pyrene substrate are generally much smaller than the effects reported in the preceding paper using the U/A duplex DNA, reflecting the pyrene rescue (*8*). Pyrene rescue effects greater than unity indicate a beneficial effect of pyrene manifested as either an increase in the on-rate or a decrease in the off-rate or K_D as compared to that of the U^F/A substrate (see Table 2 in the preceding paper) (*8*). The pyrene rescue is defined as the ratio of the measured parameter for the U^F/Y substrate divided by that for the U^F/A substrate. The effectiveness of pyrene for rescuing a given mutation may be evaluated by comparing the magnitude of the rescue with the mutational effect reported in Table 2 of the preceding paper (*8*). The average error for all the mutational and rescue effects is about ±14%; the largest errors are less than ±30%.

A U^F/Y Base Pair Enhances Uracil Recognition by Wild-Type UDG. Rapid kinetic studies of U^F/Y binding to wtUDG were also performed (Figure 3). Binding of UDG to U^F/Y elicits a large increase in pyrene fluorescence (Figure 3A), and the pseudo-first-order rate constants were fitted to the hyperbolic binding equation (eq 6) of the preceding paper (*8*): $k_{max} = 1000 \pm 170\ s^{-1}$, $k_{on} = 520 \pm 100\ \mu M^{-1}\ s^{-1}$, $K' = 0.52 \pm 0.15$, and $k_{off} = 9 \pm 5\ s^{-1}$ (Figure 3D). A trapping experiment was also used to measure the off-rate, and this approach yielded a value similar to that of the fit to eq 6 (see Table 1, Figure 3C, and the solid circle in Figure 3D). As determined in the preceding paper, we show the best-fit curves for binding of wtUDG to PU^F/A and AU^F/A DNA as dashed and dotted lines in Figure 3D for comparison with

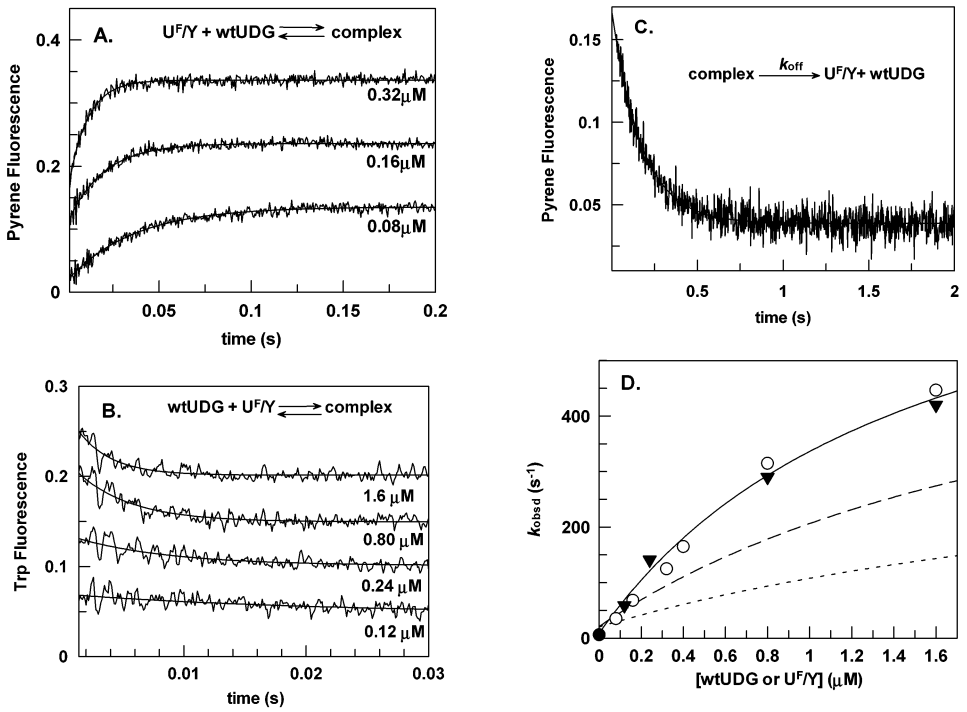

FIGURE 3: Stopped-flow fluorescence kinetic studies of DNA association and dissociation from wtUDG. (A) Approach-to-equilibrium association measurements where 100 nM U^F/Y DNA was mixed with the indicated concentrations of UDG and the pyrene fluorescence increase was monitored with a 360 nm cutoff filter with excitation at 335 nm. The lines are the fits to a first-order rate equation. (B) Approach-to-equilibrium association measurements where 30, 60, 200, and 400 nM UDG was mixed with the indicated concentrations of U^F/Y DNA and the tryptophan fluorescence decrease was monitored with a 320 nm cutoff filter with excitation at 300 nm. The lines are fits to a first-order decay rate equation. (C) Irreversible dissociation of U^F/Y DNA from UDG. A solution of 100 nM U^F/Y DNA and 0.5 μM UDG was mixed with a 10 μM concentration of an ssAU^F 11-mer nonfluorescent DNA trap to prevent reassociation of the enzyme to U^F/Y. The time-dependent decrease in 2-AP fluorescence was measured. (D) Observed rate constants from (A) (○) and (B) (●) against [wtUDG] or [U^F/Y]. The curve is a best fit to eq 6. For comparison, the dashed and dotted lines are the theoretical curves obtained from the 2-AP and tryptophan measurements using the natural U^F/A substrate analogue. The parameters are reported in Table 1.

the U^F/Y analogue (*8*). As can be seen from comparison of these curves, the U^F/Y base pair enhances the rate of DNA association, increases k_{max}, and increases the lifetime of the closed complex.

What step of the base-flipping process does the pyrene fluorescence increase detect? In many respects pyrene behaves like 2-AP, because its fluorescence is dramatically quenched when it becomes stacked with the π electron systems of the normal DNA bases. However, because little is known about the nature of pyrene fluorescence in a duplex DNA context, it is possible that the large 10-fold increase in pyrene fluorescence upon UDG binding could reflect the base-flipping step or, alternatively, the subsequent conformational docking step. To address this question, we repeated the stopped-flow binding measurements with U^F/Y except that we followed the decrease in tryptophan fluorescence of wtUDG (Figure 3B). (These experiments were performed at an excitation wavelength of 300 nm, which minimizes the excitation of the pyrene fluorophore.) The rate constants obtained from these experiments are plotted in Figure 3D as triangles for comparison with those obtained from monitoring pyrene fluorescence (open circles). Using the U^F/Y analogue, there is no discernible difference between the observed rates for the conformational change and the process that is detected as a pyrene fluorescence increase. Since both events are significantly faster than the 2-AP and tryptophan changes observed with the U^F/A base pair in the preceding paper (*8*), pyrene must have accelerated the UDG conformational change. We have previously shown using permanganate hypersensitivity measurements that the pyrene wedge preorganizes the uracil base in an extrahelical state in the free DNA (*6*). Thus, it seems likely that the encounter step with U^F/Y involves direct recognition of the extrahelical base, followed by a rapid conformational step that gives rise to the pyrene and tryptophan fluorescence changes. Consistent with this interpretation, the observed on-rate with U^F/Y is similar to the simulated k_1 value for formation of the nonspecific encounter complex with the U^F/A analogue (k_1 = 220 μM s^{-1}; see Table 3 of the preceding paper) (*8*).

Can Pyrene Rescue the Kinetic Defects of Removing the Pinch, Push, Plug, and Pull Groups? Remarkably, rapid kinetic studies showed that the damaging effects of removing the Leu191 wedge side chain are essentially fully rescued when the U^F/Y substrate analogue is used (Figure 4A−C and Tables 1 and 2). The rate constants for the tryptophan fluorescence changes were indistinguishable from the rate constants derived from the pyrene fluorescence changes, indicating that the pyrene wedge has accelerated all steps that precede the step or steps that give rise to these fluorescence changes. The linear concentration dependence shown in Figure 4C shows that the bimolecular association step is rate-limiting for L191A in the concentration range investigated. Therefore, the subsequent conformational steps are even faster and kinetically transparent. Pyrene also rescues the off-rate defects of L191A and L191G such that k_{off} matches that of wtUDG with the U^F/A analogues (compare the values in Table 1 with those in Table 2 of the preceding paper) (*8*). The observation that pyrene can rescue

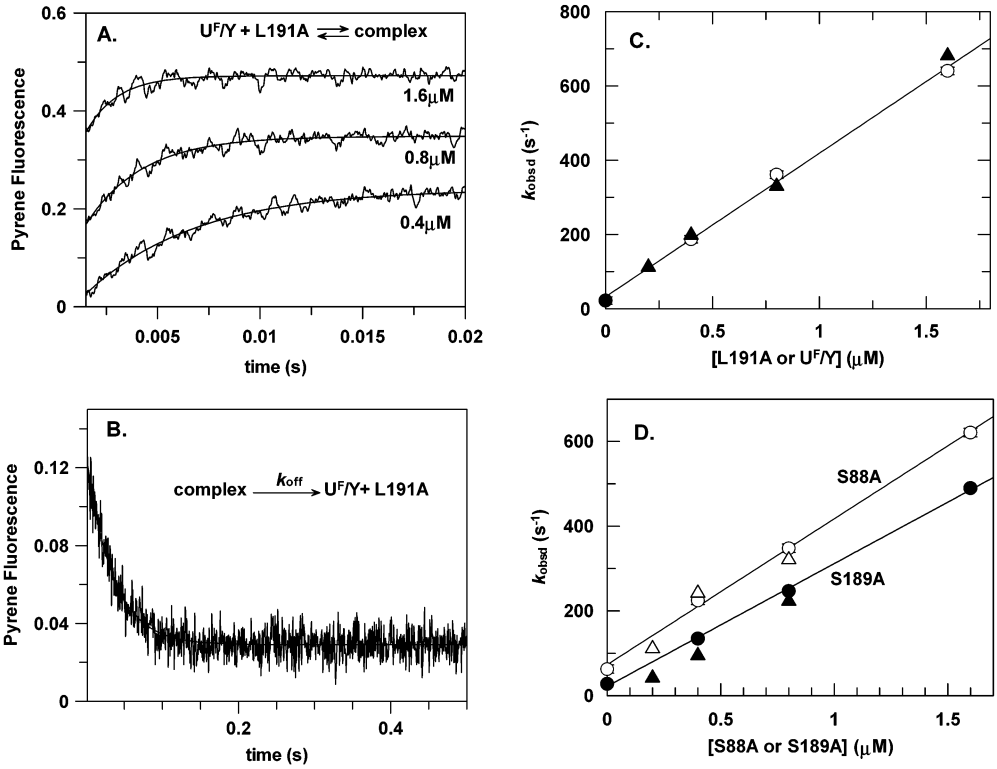

FIGURE 4: Stopped-flow fluorescence kinetic studies of DNA association and dissociation from L191A, S88A, and S189A. (A) Approach-to-equilibrium association measurements where 100 nM U^F/Y DNA was mixed with the indicated concentrations of L191A and the pyrene fluorescence increase was monitored with a 360 nm cutoff filter with excitation at 335 nm. The lines are the fits to a first-order rate equation. (B) Irreversible dissociation of U^F/Y DNA from L191A. A solution of 100 nM U^F/Y DNA and 0.5 μM L191A was mixed with a 10 μM concentration of an ssAU^F 11-mer nonfluorescent DNA trap to prevent reassociation of the enzyme to U^F/Y. The time-dependent decrease in pyrene fluorescence was measured. (C) Observed rate constants from (A) (○) and (B) (●) against [L191A]. The curve is a best fit to eq 5. The triangles correspond to the observed association rate constants measured by following the tryptophan fluorescence decrease of L191A upon U^F/Y binding. (D) Observed rate constants for U^F/Y binding to S88A and S189A. The triangles correspond to the observed association rate constants measured by following the tryptophan fluorescence decrease of L191A upon U^F/Y binding. The curve is a best fit to eq 5. The parameters are reported in Table 1.

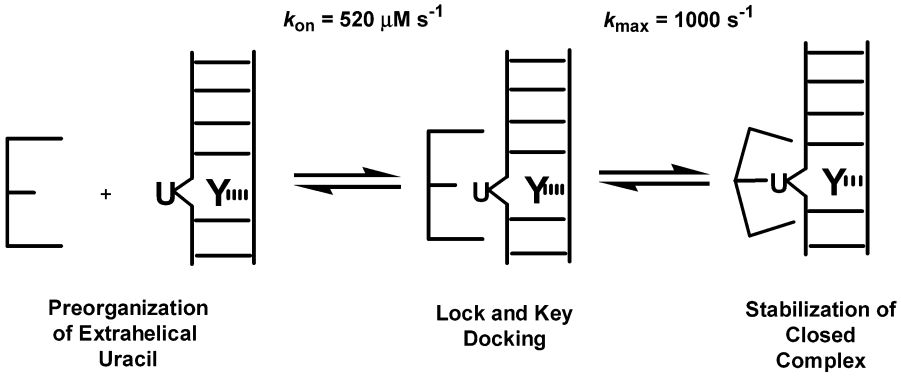

FIGURE 5: Two-step mechanism for binding of the U^F/Y substrate analogue to wtUDG. Preorganization of the target uracil in an extrahelical conformation in the free DNA leads to diffusion-controlled site-specific recognition by a lock-and-key mechanism. This step differs from recognition of a normal U/A base pair, because, for a U/A duplex, the initial encounter step forms a weak nonspecific complex in which the uracil is still stacked in the duplex (see the preceding paper in this issue) (*8*). The second step, which is also accelerated by pyrene, involves conformational closing of the enzyme around the extrahelical base. Thus, the pyrene wedge enhances recognition in two general ways: (i) by increasing the concentration of the reactive extrahelical conformation and (ii) by increasing the rate of the conformational docking step. These effects account for the rescues of the pinch, push, pull, and plug base-flipping mutations.

the association and off-rate defects of the Leu191 deletion mutants supports the proposal that Leu191 plays a role in the early and late stages of the flipping process—acting as a pushing wedge to form the extrahelical state, and a plug to increase its lifetime in the active site pocket.

Pyrene shows a distinctly different rescue effect for the serine pinching mutants as compared to the leucine push−plug mutants. The serine on-rate defects are fully rescued with the pyrene wedge, and the observed rate constants are identical whether the tryptophan or pyrene fluorescence changes are followed (Figure 4D, Tables 1 and 2). These on-rate rescues quantitatively account for the equilibrium rescues of these serine mutations, supporting the proposal that preorganizing the uracil in a productive conformation in the free DNA is the primary basis for rescue of the serine mutational effects on the binding equilibrium. In contrast

with the Leu191 mutations, the 4- and 10-fold off-rate defects of the S88A and S88A:S189A mutations cannot be rescued by pyrene because the pyrene wedge is not competent to fulfill the stabilizing interactions of these groups in the docked state. This is expected since the natural leucine wedge is already present for these mutants.

Although in the preceding paper (*8*) we were unable to measure the association kinetics of the N123G pulling mutant with the PU^F/A analogue, we were able to perform such studies with the U^F/Y analogue due to the much larger signal (Table 1). Deletion of Asn123 decreases the on-rate for U^F/Y by only 2-fold, yet increases the off-rate by 67-fold as compared to those of wtUDG. The increased off-rate probably reflects extreme destabilization of the final closed conformation of UDG. These findings strongly suggest that the hydrogen bonds of Asn123 with the uracil base form very late in the flipping process (see Figure 7 of the preceding paper) (*8*), perhaps not until the final clamping of the UDG active site around the base is complete. Consistent with this conclusion, the 16-fold weaker binding of N123G to U^F/A DNA that was reported in the preceding paper (*8*) is not rescued by pyrene (Table 2). The absence of a significant rescue of the off-rate or binding equilibrium by pyrene suggests that the final plugging by pyrene or Leu191 is not effective unless the hydrogen bonds provided by Asn123 are present to latch onto the uracil base.

CONCLUSIONS

We have shown how the normal three-step induced-fit process of damage site recognition by uracil DNA glycosylase can be reduced to a virtual lock-and-key mechanism using a conformationally preorganized substrate (Figure 5). Is the pyrene rescue approach general? Since mismatch-specific DNA repair glycosylases that recognize T/G and A/G mispairs also use wedge groups to help extrude damaged bases from DNA (*15*, *16*), it may be possible to direct these enzymes to specific T and A bases by first introducing mutations that remove the enzymatic wedge, and then targeting the enzyme to specific sites that have T/Y or A/Y base pairs. We have recently used directed mutagenesis and pyrene rescue to convert UDG into a highly specific C/Y DNA glycosylase (*17*). We are optimistic that other DNA glycosylases may allow similar engineering of their specificities using this approach.

SUPPORTING INFORMATION AVAILABLE

An improved procedure for the synthesis of pyrene nucleoside phosphoramidite. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Toney, M. D., and Kirsch, J. F. (1989) *Science 243*, 1485−8.
2. Toney, M. D., and Kirsch, J. F. (1992) *Protein Sci. 1*, 107−19.
3. Mosi, R., He, S., Uitdehaag, J., Dijkstra, B. W., and Withers, S. G. (1997) *Biochemistry 36*, 9927−34.
4. Ly, H. D., and Withers, S. G. (1999) *Annu. Rev. Biochem. 68*, 487−522.
5. Wang, Q., and Withers, S. G. (1995) *J. Am. Chem. Soc. 117*, 10137−10138.
6. Jiang, Y. L., Kwon, K., and Stivers, J. T. (2001) *J. Biol. Chem. 276*, 42347−54.
7. Matray, T. J., and Kool, E. T. (1999) *Nature 399*, 704−8.
8. Jiang, Y. L., and Stivers, J. T. (2002) *Biochemistry 41*, 11236−11247.
9. Ren, R. X. F., Chaudhuri, N. C., Paris, P. L., Rumney, S., and Kool, E. T. (1996) *J. Am. Chem. Soc. 118*, 7671−7678.
10. Jiang, Y. L., and Stivers, J. T. (2001) *Biochemistry 40*, 7710−9.
11. Moe, J. G., and Russu, I. M. (1992) *Biochemistry 31*, 8421−8.
12. Wu, P. G., Nordlund, T. M., Gildea, B., and McLaughlin, L. W. (1990) *Biochemistry 29*, 6508−14.
13. Xu, D., Evans, K. O., and Nordlund, T. M. (1994) *Biochemistry 33*, 9592−9.
14. Stivers, J. T., Pankiewicz, K. W., and Watanabe, K. A. (1999) *Biochemistry 38*, 952−63.
15. Guan, Y., Manuel, R. C., Arvai, A. S., Parikh, S. S., Mol, C. D., Miller, J. H., Lloyd, S., and Tainer, J. A. (1998) *Nat. Struct. Biol. 5*, 1058−64.
16. Barrett, T. E., Scharer, O. D., Savva, R., Brown, T., Jiricny, J., Verdine, G. L., and Pearl, L. H. (1999) *EMBO J. 18*, 6599−609.
17. Kwon, K., Jiang, Y., and Stivers, J. (2002) *Biophys J. 82*, 595.

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