Probing the Limits of Electrostatic Catalysis by Uracil DNA Glycosylase Using Transition State Mimicry and Mutagenesis*§

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The DNA repair enzyme uracil DNA glycosylase (UDG) hydrolyzes the glycosidic bond of deoxyuridine in DNA by a remarkable mechanism involving formation of a positively charged oxacarbenium ion-uracil anion intermediate. We have proposed that the positively charged intermediate is stabilized by being sandwiched between the combined negative charges of the anionic uracil leaving group and a conserved aspartate residue that are located on opposite faces of the sugar ring. Here we establish that a duplex DNA oligonucleotide containing a cationic 1-aza-deoxyribose (I) oxacarbenium ion mimic is a potent inhibitor of UDG that binds tightly to the enzyme-uracil anion (EU−) product complex (K_{off} of EU− = 110 pm). The tight binding of I to the EU− complex results from its extremely slow off rate (k_{off} = 0.0008 s−1), which is 25,000-fold slower than substrate analogue DNA. Removal of Asp^{64} and His^{187}, which are involved in stabilization of the cationic sugar and the anionic uracil leaving group, respectively, specifically weakens binding of I to the UDG-uracil complex by 154,000-fold, without significantly affecting substrate or product binding. These results suggest that electrostatic effects can effectively stabilize such an intermediate by at least −7 kcal/mol, without leading to anticatalytic stabilization of the substrate and products.

To provide a rigorous test of the above catalytic proposal, we have constructed a stable bipartite mimic of the charged intermediate using a cationic 1-aza-deoxyribose (I) containing DNA and uracil (Fig. 1). If the electrostatic stabilization mechanism is correct, we predict that I will bind extremely tightly to the UDG-uracil anion binary complex and that the tight binding would be abolished when either the uracil base, His^{187}, or Asp^{64} was removed by omission or mutagenesis. An additional rigorous test for the catalytic potential of this mechanism is to evaluate whether His^{187} and Asp^{64} are involved in differential stabilization of the intermediate through their interactions with the uracil base and cationic sugar as shown in Fig. 1. This requirement for catalysis may be tested by assessing whether the removal of His^{187} and Asp^{64} has a large effect on binding of I without a corresponding large effect on the binding of substrate or product analogue DNA. Here we test these predictions and report that UDG provides a large and differential electrostatic stabilization of the oxacarbenium ion-uracil anion intermediate analogue using the negative charge on the uracil anion and Asp^{64}. The magnitude of the stabilization is on the order of −7 kcal/mol, which is a significant portion of the −16 kcal/mol decrease in the activation barrier provided by UDG.

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UDG, uracil DNA glycosylase; HPLC, high-performance liquid chromatography; I, 1-aza-2-deoxy-4-o-carboxy-

The abbreviations used are: UDG, uracil DNA glycosylase; HPLC, high-performance liquid chromatography; I, 1-aza-2-deoxy-4-o-carbadoxynucleoside; UF, 2'-β-fluoro-2'-deoxyuridine nucleotide; Y, pyrene deoxyribonucleotide; MES, 2-(N-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; EU−, enzyme-uracil anion; KIE, kinetic isotope effect.
Electrostatic Stabilization of an Oxacarbenium Ion

**Experimental Procedures**

Nucleoside Phosphoramidite and Oligonucleotide Synthesis—The nucleoside phosphoramidites were purchased from Applied Biosystems or Glen Research (Sterling, VA), except for the β anomer of the pyrene nucleoside phosphoramidite, the 2-β-fluoro-2-deoxyuridine phosphoramidite, and the 1-aza-1,2-dideoxy-α-carba-ribitol phosphoramidite, which were synthesized as described (12–14). The oligonucleotides were synthesized using standard phosphoramidite chemistry with an Applied Biosystems 390 synthesizer. After synthesis and deprotection, the oligonucleotides were purified by anion exchange HPLC and desalted by C-18 reversed phase HPLC (Phenomenex Aqua column) (Torrance, CA). The size, purity, and nucleotide composition of the DNA was assessed by analytical reversed phase HPLC, matrix-assisted laser desorption ionization mass spectrometry, and denaturing polyacrylamide gel electrophoresis. The DNA strands were hybridized as previously described to form the duplexes used in the binding studies as shown in Figure 2. The concentrations of the oligonucleotides were determined by UV absorption measurements at 290 nm, using the pair wise 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.

**Purification of UDG and Mutants—**As previously described, the recombinant UDG from Escherichia coli strain B was purified to >99% homogeneity using a T7 polymerase-based over expression system (6, 15). The concentration of the enzyme was determined using an extinction coefficient of 38.5 mM⁻¹ cm⁻¹. The D64N and H187G mutants were generated using the QuikChange double-stranded mutagenesis kit from Stratagene (La Jolla, CA), and the mutations were confirmed by sequencing both strands of the DNA. The His₆-tagged mutant proteins were purified using nickel chelate chromatography as previously described (6, 16). The His₆-tag was removed by cleavage using biotinylated thrombin followed by purification using streptavidin beads and nickel chelate chromatography.

**pKₐ Determination of 1-Aza-deoxyribose by Proton NMR—**One-dimensional proton NMR experiments were performed at 25 °C with a Varian mercury 400 MHz NMR spectrometer (Palo Alto, CA). The sample included 2 mM 1-aza-deoxyribose and the internal standard p-toluene sulfonic acid in 1 mM D₂O. The spectra were recorded with the following acquisition parameters: a spectral width of 6390 Hz, a reference frequency set at 0 ppm relative to the methyl group of the standard, an acquisition time of 2 s, and a relaxation delay of 1 s. Titrations were performed by adding small aliquots of 0.1 M NaOD or DCI. The pKₐ value for the endocyclic nitrogen of the 1-aza-deoxyribose was determined by following the pH-dependent chemical shifts of the adjacent 2' or 4' protons (17). The apparent electrode readings were not corrected for deuterium isotope effects, because the glass electrode effect is expected to approximately cancel the increased pKₐ of the phosphate groups in D₂O (18). This may introduce an uncertainty of ±0.1 unit in the true group pKₐ value in water, but this uncertainty has no impact on the conclusions in this work. The titration data were fitted by nonlinear regression analysis to Equation 1, where δ₁ and δ₂ are the limiting chemical shifts at low and high pH, respectively.

\[
\delta(ppm) = \delta_1 + \delta_2(10^{\text{pH}} - \delta_1)/(10^{\text{pH}} - \delta_2) + 1
\]  

**Binding and Competitive Inhibition Studies—**The dissociation constants (K_D) for binding of UDG to the DNA molecules indicated in Table I were determined using three methods that we designate as methods A, B, and C. All measurements were performed in 10 mM Tris-HCl (pH 8.8), 2.5 mM MgCl₂, 25 mM NaCl at 25 °C. With method A, direct binding measurements were made by following the increase in pyrene fluorescence upon titrating fixed concentrations of the pyrene-containing DNA (Fig. 2) 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 spectrophotometer (19). The fluorescence intensity (F) at 380 nm was plotted against [UDG]tot to obtain the K_D from Equations 2 and 3.

\[
F = F_o - [(F_o - F_i)/(DNA)]_{obs}(b - (b^2 - 4UDG)_o(DNA)_{obs})^{1/2}
\]

(Eq. 2)

\[
b = K_D + [UDG]_{tot} + [DNA]_{tot}
\]

(Eq. 3)

To measure the affinity of A/TYT or Aα/A/TYT to the UDG-uracil anion binary complex, the titrations included a saturating concentration of uracil (3 mM), and the titration was performed at pH 8.8. Further experimental details are reported in the figure legends. With method B, which was used for measuring the binding of A[U₄/A/TYT to wtUDG, competitive kinetic inhibition measurements were performed using the substrate ApUpAp (19). Conditions were chosen whereby [UDG]tot << [A[U₄/A]/TYT] and [ApUpAp], and [ApUpAp] << K_D. Accordingly, K_D could be obtained directly from a plot of k/off against [A[U₄/A/TYT] as shown in Equation 4, where k is the observed rate constant ([UDG]tot) at a given [A[U₄/A/TYT], and k_D is the observed rate constant in the absence of the inhibitor.

\[
k/off = 1/(1 + [A[U₄/A]/TYT]/K_D)
\]

(Eq. 4)

For these measurements, a sensitive HPLC kinetic assay for monitoring the formation of the abasic product was employed (20). With method C, the K_D of A/TYT for the wtUDG-uracil anion complex was calculated from the ratio of k_on to k_off, which was obtained from stopped flow and steady state fluorescence measurements (see below).

**Fluorescence Measurements of the Association and Dissociation Rate Constants—**The observed rate constants for association of A/TYT with the UDG-uracil anion complex were obtained using an Applied Photophysics 720 stopped flow fluorescence instrument (Surrey, UK) under pseudo-first-order conditions in which the concentration of the EU⁺ complex was always more than 4-fold greater than the concentration of AIA/TYT. In these experiments a syringe containing a solution of UDG (200–1800 nM) and uracil (3 mM) was rapidly mixed with a solution of AIA/TYT (50 nM) delivered from a second syringe, and the fluorescence change as a function of time was recorded using a 360-nm cut-off filter with excitation at 333 nm. The traces were fitted to a first-order rate expression (Equation 5) to obtain the observed rate constants (k_on) at each concentration of UDG.

\[
k_on = A \exp(1 - kt) + C
\]

(Eq. 5)

The k_on values were plotted against the concentration of the UDG-uracil anion complex, and the association rate was obtained from the slope of a linear regression best fit line to the data. The dissociation rate constant (k_off) of AIA/TYT from the UDG-uracil anion complex was measured using irreversible conditions by manually mixing the complex with a large excess of nonfluorescent single-stranded trapping DNA and then following the time-dependent decrease in the pyrene fluorescence at 378 nm. The sequence of the trap DNA was the same as the AIA strand of the duplex AIA/TYT (Fig. 2). The experiments were performed by first incubating UDG (50 nM), AIA/TYT (40 nM), and uracil (3 mM) in the fluorescent cuvette for 60 min and then initiating the reaction by adding 2 μl of a 16.7 mM stock solution of AIA. The solution was mixed, and the fluorescence at 378 nm was monitored for 4000 s.

**pH Dependence of Binding—**The pH dependence of AIA/TYT binding...
to the EU complex was determined using the direct fluorescence method (method A, see above) in the pH range 6–10 using the following buffers (all 10 mM containing 2.5 mM MgCl₂ and 20 mM NaCl): NaMES at pH 6.0, NaHEPES at pH 7 to 8, Tris-HCl at pH 8.8, and NaCAPS at pH 9.3 and 10. The uracil concentration was maintained at 3 mM so that the enzyme was nearly entirely in the EU form at all pH values (8).

RESULTS

Overall Experimental Design and Rationale—The primary purpose of the current studies is to evaluate the magnitude and mechanism by which UDG stabilizes the putative oxacarbenium ion-uracil anion intermediate that was implicated in recent KIE studies (10). The experimental design involves measurement of the binding affinity of an oxacarbenium ion mimic to the UDG-uracil anion complex, as well as the uracil complex with the H187G and D64N mutants. The DNA construct contains a pyrene nucleotide fluorescent reporter group on the DNA strand opposite to the 1-aza-deoxyribose group. We have previously shown that pyrene (Y) has a slight beneficial effect on the kinetics of the wild-type UDG reaction and increases the binding affinity for the substrate by about 5-fold because of preorganization of the uracil base in an extrahelical conformation (19). Most importantly for the work presented here, pyrene provides a strong fluorescence signal to monitor binding (19).

Interpretation of the binding and mutagenesis results requires knowledge of the ionization states of the important residues of the enzyme, the free and bound uracil base, and the free and bound 1-aza-deoxyribose oxacarbenium ion mimic. Fortunately, thermodynamic, kinetic, and NMR studies of UDG provide most of the required information to design the current experiments without making assumptions about the ionization states of any of the free or bound species. We present our results by first establishing the ionization states of each key species and then describing the binding thermodynamics for the wild-type, H187G, and D64N enzymes.

Ionization State of Uracil Bound to Wild-type, H187G, and D64N UDG—The N-1 \( \beta \) value of 9.4 ± 0.03 in D₂O was determined from the best fit to Equation 1. The chemical shifts were referenced to internal p-toluenesulfonic acid.

to the EU complex was determined using the direct fluorescence method (method A, see above) in the pH range 6–10 using the following buffers (all 10 mM containing 2.5 mM MgCl₂ and 20 mM NaCl): NaMES at pH 6.0, NaHEPES at pH 7 to 8, Tris-HCl at pH 8.8, and NaCAPS at pH 9.3 and 10. The uracil concentration was maintained at 3 mM so that the enzyme was nearly entirely in the EU form at all pH values (8).

FIG. 2. Substrate, intermediate, and product analogues for UDG used in this study. The pyrene (Y) nucleotide that is located on the DNA strand opposite to \( \text{UF}^\circ \), I, and \( \phi \) provides a strong fluorescence signal for monitoring binding to UDG (19). As discussed in previous work, duplex DNA containing a \( \text{UF}^\circ \):Y base pair binds 5-fold more tightly to UDG as compared with the same DNA with a \( \text{UF}^\circ \):A base pair because pyrene pushes the \( \text{UF}^\circ \) into an extrahelical conformation. In addition, Y:U-containing DNA has a 3-fold greater \( k_{\text{cat}}/K_m \) as compared with DNA containing a U:A base pair (19). These modest effects of pyrene have no influence on the comparisons here because pyrene is present in all of the DNA constructs.
The $K_D$ measurement methods correspond to direct fluorescence titration (A), competition (B), and the ratio method (C). If the ionization state of uracil is not known, it is simply indicated as "U." 1-aza-2-dideoxy-4α-carba-γ-deoxyribo-
nucleotide; $\text{U}^\gamma$, 2'-β-fluo-2'-deoxyuridine nucleotide; $\text{Y}$, pyrene deoxyribonucleotide.

| Analogue and enzyme tested | Interaction mimicked | $K_D$ (nM) | Measurement method |
|---------------------------|----------------------|------------|-------------------|
| wtUDG                     | E + S = E-S          | 31 ± 2     | B                 |
| AIA/TT                   | E'U' + I = E'U'-I    | 0.11 ± 0.01| C                 |
| AIA/TT                   | E + I = E-I          | 2,500 ± 400| A                 |
| AIA/TT                   | E'U' + P = E'U'-P    | 19,000 ± 4,000| A |
| AIA/TT                   | E + P = E-P          | 2,400 ± 360| A                 |
| H187G                    | A/TT                 | 48 ± 2     | A                 |
| A/TT                     | E'UH + I = E'UH-I    | 35 ± 5     | A                 |
| A/TT                     | E'UH + P = E'UH-P    | 30,000 ± 11,000| A |
| D64N                     | A/TT                 | 9 ± 1      | A                 |
| D64N                     | E + S = E-S          | 13 ± 2     | A                 |
| D64N/H187G               | E'U' + P = E'U'-P    | 1,400 ± 300| A                 |
| D64N/H187G               | E + S = E-S          | 103 ± 12   | A                 |
| A/TT                     | E'UH + I = E'UH-I    | 17,000 ± 2,000| A |
| A/TT                     | E'UH + P = E'UH-P    | 38,000 ± 4,000| A |

$K_D$ value of UDG for the neutral uracil base is 500-fold weaker than for the uracil anion ($K_D = 500$ and 1 μM, respectively) (8). These two results establish that at pH 8.8, the standard pH used in this work, UDG is essentially saturated with the uracil anion and not a mixture of the neutral and anionic forms. Thus the binding experiments with wild-type UDG measure the affinity of AIA/TT and A/TT for the enzyme-uracil anion complex.

Interpretation of the mutagenesis tests of the electrostatic sandwich mechanism also requires knowledge of the ionization state of the uracil base in the complexes with the H187G and D64N mutants at pH 8.8. The crystal structure of the H187G mutant bound to uracil has been solved at 1.2 Å resolution (7) and shows that the uracil base is productively bound at pH 8.5 but provides no information as to its ionization state. However, based on the weak $K_D$ of uracil for binding to H187G or H187Q, and the absence of a $2^{-13}$C shift for uracil of 165 ppm that would indicate the presence of the N-1–O-2 uracil monoanion (9), we conclude that uracil is neutral in the binary and ternary product complexes with these mutants (8). In contrast, the D64N mutant binds uracil with the same affinity as wild-type UDG (6) and retains the downfield-shifted proton in the ternary complex with abasic DNA that is diagnostic for the uracil anion (see below).

**Ionization State of 1-Aza-deoxyribose**—To establish whether I is a true oxacarbenium ion mimic, it is essential to establish its ionization state. The $pK_a$ value for the 1-nitrogen of the free 1-aza-deoxyribose (I) was determined using proton NMR by following the pH dependence of the chemical shift of the pro- $R$ endocyclic 4 proton that is adjacent to the nitrogen ($pK_a = 9.42 ± 0.03$; Fig. 3). An identical $pK_a$ value was measured by following the 2' proton resonances as well. Thus in the pH range 6.5–8.8 where the bulk of the current binding measurements are performed, free I is at least 80–100% in its cationic form. For comparison, the $pK_a$ value of isofagomine, the related 1-aza-pyranose, is 0.8 units lower than the 1-aza-furanose used here ($pK_a = 8.6$) (21). The $pK_a$ of I in the context of DNA is likely to be even higher because of electrostatic stabilization of the cation by DNA phosphodiester groups. On the basis of the high $pK_a$ of free I and the negatively charged groups in the vicinity of the anomic position in the active site (Fig. 1), we conclude that I likely remains cationic in the E'U'·AIA/TT complex.

Although we have no direct method to determine the ionization state of I in the E'U'·AIA/TT ternary complex, we can indirectly infer its protonation state by observing the effect of I on NMR observable features of this complex (8, 9). The first NMR feature is the downfield-shifted H$^*$ proton of the active site electrophile His$^{187}$. This resonance is only observed when the uracil base is in the N-1–O-2 enolate form (Fig. 1) and thus provides a useful probe of the ionization state of the bound uracil base (8, 9). In addition, this signal should indirectly report on the protonation state of I because a positive charge on I should stabilize the uracil anion and thereby lower its $pK_a$. We have previously used the decrease in intensity of the H$^*$ proton resonance as the pH is lowered to measure the $pK_a$ of uracil N-1 in a UDG ternary complex with DNA containing a ---A. C. Drohat and J. T. Stivers, unpublished results.
A.

![Pyrene Fluorescence vs Time Graph](image)

**FIG. 6.** Determination of the association and dissociation rates of AIA/TYT to the UDG-uracil anion binary complex at pH 8.8 using stopped flow and steady state fluorescence measurements. A, time course for the observed change in pyrene fluorescence as AIA/TYT (50 nM) binds to the UDG-uracil anion complex (800 nM). The experiment was performed by rapidly mixing a solution containing UDG and uracil with an equal volume solution of AIA/TYT. At pH 8.8 the uracil base is essentially completely bound to the enzyme as the uracil anion (8); thus the experiment measures binding of AIA/TYT to the binary complex. The final concentrations were 800 nM UDG, 3 mM total uracil, and 50 nM AIA/TYT. The data were fitted to a first-order rate equation to obtain $k_{\text{on}} = 6.6 \text{ s}^{-1}$. B, linear plot of the observed rate constants for AIA/TYT binding against the concentration of the UDG-uracil anion complex after mixing. The slope of the best fit line provides $k_{\text{on}} = 7.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. C, irreversible dissociation kinetics of AIA/TYT from the UDG-uracil anion complex. The time-dependent decrease in the pyrene fluorescence of AIA/TYT was followed after the addition of a neutral tetrahydrofuran abasic site analogue ($pK_a = 6.4$) (8). Importantly, the $pK_a$ determined by this method was identical to that measured in NMR experiments in which the 2-13C chemical shift of 2-13C-labeled uracil was followed as a function of pH (9).

We performed the same one-dimensional proton NMR experiment with the E-U-AIA/TAT complex and observed no decrease in the intensity or change in shift of the $H^+$ proton resonance as the pH was decreased from 10.1 to 5.6, and data in the range pH 8.8–5.6 are shown in Fig. 4. For comparison, the decrease in intensity of the $H^+$ proton resonance in the E-U-AIA/TAT complex over a similar pH range is also shown. The absence of an intensity change of the $H^+$ proton resonance in the E-U-AIA/TAT complex suggests that the uracil base is still fully anionic even at pH values as low as 5.6 with this complex. This result suggests that the positive charge on I has dramatically lowered the $pK_a$ of uracil N-1 as compared with the ternary complex with the neutral abasic site analogue (Fig. 2). On the basis that no intensity change of the $H^+$ resonance was observed at pH 5.6, we calculate an upper limit $pK_a$ for uracil N-1 of less than 4.5 in this complex. This corresponds to a $\geq 5.2$ unit lowering of the $pK_a$, as compared with free uracil ($pK_a = 9.8$) or, alternatively, a $\geq 7.5$ kcal/mol stabilization of the uracil anion in the active site environment. Furthermore, the observation that the resonance at 15.15 ppm did not change in shift (<0.06 ppm) over the pH range 5.6–10.1 suggests that I remains cationic over this pH range. This interpretation is supported by the sensitivity of this shift to mutation and DNA structure, which makes it unlikely that a change in the ionization state of I would escape detection (8). Further strong evidence that I is cationic in the E-U-AIA/TYT complex is provided by the selective tight binding of AIA/TYT to the EU–complex, which can only reasonably be explained by the presence of positive charge on the sugar (see below).

**Ionization State of Asp$^{64}$—**The electrostatic sandwich mechanism requires a negative charge on Asp$^{64}$. The $pK_a$ value of Asp$^{64}$ in the free enzyme and ES complex has been inferred from kinetic and mutagenesis studies to be 5.7 and 6.2, respectively (6). Thus it would be expected that binding of AIA/TYT would weaken as the pH is lowered below 6.2 because of a weaker interaction with the neutral form of Asp$^{64}$. We do not have any method of determining the $pK_a$ value of Asp$^{64}$ in the E-U-AIA/TYT complex, but we anticipate that its $pK_a$ would be lower than 6.2 because of electrostatic stabilization of the anion by the protonated nitrogen of the sugar (see “Discussion”).

**Binding of Substrate, Intermediate, and Product Analogues at pH 8.8—**Using method A, B or C described under “Experimental Procedures,” we have determined the binding constant of AU$^3$/TYT substrate analogue DNA to free UDG and the binding constants of the intermediate and product analogue DNA molecules to the UDG-uracil anion complex (Table I). A representative binding experiment is shown in Fig. 5 in which a large excess of the nonfluorescent AIA-11 trap DNA (220 nM). The concentrations of AIA/TYT and UDG were 40 and 50 nM, respectively, and the concentration of uracil was 3 mM. The data were fitted to a single exponential decay equation, which provided $k_{\text{off}} = (8 \pm 0.1) \times 10^{-4} \text{ s}^{-1}$.
Fig. 7. Binding affinities of wtUDG, H187G, D64N, and D64N/H187G for the substrate, intermediate, and product analogues shown in Fig. 2 at pH 8.8. The top panel compares the binding of the wild-type and mutant enzymes with the AUφA/TYT substrate analogue. The middle panel compares the binding of each enzyme to the intermediate analogue AIA/TYT in the presence of a saturating concentration of uracil (3 μM). Depending on the enzyme, the measurement reflects binding of AIA/TYT to the enzyme-uracil anion binary complex (D64N and wtUDG) or the enzyme-neutral uracil complex (H187G and H187G/D64N) (see text). The bottom panel compares the binding of each enzyme to the product analogue, AφA/TYT-11, in the presence of a saturating concentration of uracil (3 μM). The measurements were made by one of three methods as described under “Experimental Procedures.”

a solution of AIA/TYT (12 nM) and uracil (3 μM) is titrated with increasing concentrations of wtUDG, which results in a saturable increase in pyrene fluorescence. The $k_{off}$ obtained from this experiment measures the binding of AIA/TYT to the UDG-uracil anion binary complex.

Because of the very tight binding of AIA/TYT to the binary complex and the signal-to-noise limitations of the fluorescence assay, we were unable to obtain a more precise measurement of this interaction using this direct titration approach. Therefore, to confirm this $K_D$ estimate, we performed kinetic measurements of the association and dissociation rates using stopped flow and manual mixing methods and then calculated $K_D$ from the ratio $k_{off}/k_{on}$ (Fig. 6 and Table I). The observed rate constants for association of AIA/TYT to the EU-complex were measured using four concentrations of UDG over the range 200–1600 nM (Fig. 6B) and $k_{on} = (7.5 ± 0.2) \times 10^8$ M$^{-1}$ s$^{-1}$ was determined from the slope of a linear regression fit of $k_{on}$ against UDG concentration (see legend to Fig. 5 for further details). The $k_{off} = (8 ± 0.1) \times 10^{-4}$ s$^{-1}$ was then measured in a trapping experiment in which irreversible dissociation of AIA/TYT from the ternary complex was monitored by the decrease in pyrene fluorescence (Fig. 6C). This off rate is 25,000-fold slower than the previously measured $k_{off} = 20$ s$^{-1}$ for a substrate analogue DNA (13). The ratio, $k_{off}/k_{on} = 110 ± 10$ ps, obtained from these kinetic measurements overlaps the estimated $K_D$, obtained from the titration experiment in Fig. 5 and confirms the tight binding of this analogue to the EU-complex. We also investigated the binding affinity of AIA/TYT to the free enzyme in the absence of uracil using method A. The $K_D = 2500$ nM for this interaction is 25,000-fold weaker than binding of AIA/TYT to the EU-complex, confirming the expectation that the complete ternary complex is required to realize the full binding affinity of the intermediate analogue. As shown in Table I, the $K_D$ of AIA/TYT for the EU-complex is 300-fold tighter than AUφA/TYT binding to the free enzyme and an enormous 172,000-fold tighter than the binding affinity of AφA/TYT to the EU-complex. This is remarkable given that the major difference between AIA/TYT and AφA/TYT is the replacement of the 1-methylene group with NH$_2$.

Fig. 8. pH dependence of AIA/TYT binding to the UDG-uracil complex. The dissociation constants were determined using method A or C (see “Experimental Procedures”). Lines with slopes of one and two are shown for reference. As the pH is lowered, the uracil in the EU complex becomes protonated (pK$_a = 7.5$) (8) and perhaps Asp$^{64}$ as well, leading to the observed second-order dependence of log $K_D$ on proton concentration below pH 7. At pH values greater than 9 in this titration, the protons attached to the 1-nitrogen of I (pK$_a = 9.4$), and the 1- and 5-nitrogens of uracil (free pK$_a = 9.0$) may be removed, which would also lead to a second-order dependence of log $K_D$ on proton concentration in this pH range. The sharp pH dependences of the apparent binding constants at low and high pH values are consistent with the electrostatic sandwich mechanism. We have not attempted to fit the pH dependence of the apparent $K_D$ values to a mechanism, because such a fit would require calculation of the pK$_a$ values of free and bound uracil and I (in the context of DNA), free and bound Asp$^{64}$, and the K$_D$ values of neutral and protonated AIA/TYT for each protonation state of the enzyme-uracil complex.

Energetic Effects of Deleting His$^{187}$ and Asp$^{64}$—To investigate the hypothesis that His$^{187}$ and Asp$^{64}$ serve to selectively stabilize the oxacarbenium ion-uracil anion intermediate without also contributing to the binding of the substrate and product, we measured the binding affinity of H187G, D64N, and H187G/D64N to the substrate, intermediate, and product analogues (Table I). As shown in Fig. 7, the three mutants bind with similar affinity as wtUDG to the substrate analogue AUφA/TYT. In addition, all of the mutants bind to the product analogue AφA/TYT with affinity similar to that of wtUDG in the presence of 3 μM uracil. However, H187G and D64N bind 100- and 300-fold less tightly than wtUDG to the AIA/TYT intermediate analogue in the presence of 3 μM uracil, and the H187G/D64N double mutant binds 154,000-fold less tightly. The excellent agreement between the damaging effects of removing the positive charge on the sugar (i.e. binding of AφA/TYT to the EU-complex) and the deletion of both Asp$^{64}$ and His$^{187}$ (i.e. binding of AIA/TYT to the double mutant) provides two independent measurements supporting the magnitude of electrostatic stabilization of the intermediate by wtUDG.

pH Dependence of AIA/TYT Binding to the EU Complex—
The pH dependence of binding to form the E-U-AIA/TYT ternary complex would be expected to be extremely complex, reflecting the pK$_a$ values of the free and bound forms of uracil, AIA/TYT, His$^{187}$, and Asp$^{64}$. Despite this complexity, which precludes a rigorous analysis, we measured the apparent binding constants at six pH values in the range 6.0–10 (Fig. 8). The apparent $K_D$ for binding decreased with a roughly second-order dependence on proton concentration in the pH range 6.0–7.0, suggesting that protonation of two groups in this pH range was important for binding. As the pH was further increased from 7 to 9, the $K_D$ decreased with a first-order dependence before increasing sharply as the pH was raised above 9.3 (Fig. 8). This pH dependence is consistent with electrostatic sandwich mech-
Electrostatic Stabilization of an Oxacarbenium Ion

The specificity of the His187 interaction with the intermediate is electrostatic interaction with the cationic sugar. Importantly, effects in the transition state and electrostatic stabilization by sugar pucker that maximizes stabilizing hyperconjugative effects of the upper limit energetic contributions of these groups measured in recent experimental studies (6.0–9.5 kcal/mol) (6).

The UDG Active Site Is Tailored to Stabilize a Dissociative Transition State and Unstable Oxacarbenium Ion Intermediate—The small measured 1H KIE of UDG (1.01) and the large 1-α-δ (1.2) and 2-β-δ kinetic isotope effects (1.1) led to the conclusion that the UDG reaction followed one of the most dissociative mechanisms yet observed, with the likely formation of a discrete oxacarbenium ion-uracil anion intermediate (10). A subsequent computational study reached the same conclusion (22). Features of the active site that contributed to this surprising structure were the use of binding interactions with the 3′- and 5′-phosphodiester groups to enforce a flattened sugar pucker that maximizes stabilizing hyperconjugative effects in the transition state and electrostatic stabilization by Asp64 and the uracil anion leaving group. The present results are fully supportive of these proposals and provide estimates of the amount of specific stabilization of the intermediate that may be afforded by Asp64 and the uracil anion. The current study cannot address the magnitude of transition state stabilization provided by electrostatic interactions of the intermediate with the phosphodiester groups of the substrate. A recent computational study suggests that these effects may contribute an astonishing 22 kcal/mol to catalysis, which is in great excess of the upper limit energetic contributions of these groups measured in recent experimental studies (<13 kcal/mol) (20). The role of the anionic phosphodiester groups in stabilization of the intermediate and transition state is under further investigation.

The removal of Asp64 is expected to selectively impair binding of AIA/TYT to the EU complex by abolishing the favorable ionic interaction with I. The apparent magnitude of this single interaction is 2.9 kcal/mol on the basis of the 118-fold damaging effect of removing Asp64 (Table I). The specificity of this interaction for the intermediate mimic and not the substrate and product is demonstrated by the observation that the D64N mutation actually increases the binding affinity for the substrate and product analogues by 3.4- and 21-fold, respectively. We have noted this effect of the D64N mutation before (20) and speculate that it could arise from an unfavorable electrostatic interaction between Asp64 and the 3′-phosphodiester group of the deoxyuridine residue of the substrate or the tetrahydrofuran ring of the product.

The removal of His187 is expected to selectively impair binding of AIA/TYT to the EU complex by abrogating the negative charge on the uracil base (where UH is the N-1 protonated uracil). The magnitude of this interaction is 3.4 kcal/mol on the basis of the 318-fold damaging effect of removing His187 (Table I). The specificity of the His187 interaction with the intermediate is demonstrated by the less than 1.6-fold difference in the binding affinity of H187G with the substrate and product analogues as compared with wtUDG (Table I). Consistent with approximately additive effects of the single mutations, the D64N/H187Q double mutant shows an enormous 154,000-fold detrimental effect on binding of the intermediate analogue (7.2 kcal/mol) and only a 2–3-fold effect on binding of the substrate and product analogues (Fig. 6). We conclude that electrostatic stabilization of the oxacarbenium ion intermediate involves His187, though its stabilization of the uracil anion, as well as Asp64, through its direct electrostatic interaction with the cationic sugar. Importantly, both of these interactions are catalytic by virtue of their selective stabilization of the intermediate mimic.

The Importance of Balanced pKₐ Values—For the 1-aza-deoxyribose inhibitors to be of general utility, the nitrogen must be protonated in the active site of the enzyme. The NMR titration in Fig. 3 establishes that the free I has a fairly high pKₐ value, ensuring that it is cationic at neutral pH. In the active site, its pKₐ may be even higher because of the stabilizing effects of nearby phosphodiester groups, Asp64 and the uracil anion. Similarly, the cationic I would be expected to lower the pKₐ of Asp64 and lower the pKₐ of uracil N-1, which is indeed observed (Fig. 4). Thus with the estimated pKₐ values falling in the range 4.5 (uracil), 6.2 (Asp64), and 9.4 (I) the system is set so that proton transfer from the sugar nitrogen to uracil N-1 or Asp64 is thermodynamically unfavorable. This pKₐ balance is necessary to maintain the correct ionization state of the system required for tight binding, as indicated by the pH dependence in Fig. 8.

Implications for Inhibition of Glycosylases—Specific inhibitors of uracil DNA glycosylase could serve as antiviral agents, because the pox viruses and type I herpesvirus require a UDG activity for viral DNA replication or escape from latency (23–26). In general, such inhibitors could have potential for inhibiting DNA glycosylases that counterproductively repair damaged bases that result from chemotherapy treatments with alkylating agents or radiation. To our knowledge, this is the first example where tight binding of a 1-azasugar derivative has been directly demonstrated for an enzyme that is known to proceed through a discreet oxacarbenium intermediate. If other DNA glycosylases are found to follow similar highly dissociative mechanisms as UDG, with the formation of discrete intermediates, then a general strategy for inhibitor design would be to incorporate the features of the bipartite intermediate into a unimolecular inhibitor. In the case of UDG, this would require attachment of the uracil base to the sugar in such a manner that ionization at N-1 is still possible, allowing the negative charge on the base to be retained. The syntheses of several inhibitors that may be able to mimic all of the features of the ionic intermediate are in progress, and this approach may provide a general strategy for specific inhibition of a variety of DNA repair glycosylases.

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