Presteady-state Analysis of a Single Catalytic Turnover by Escherichia coli Uracil-DNA Glycosylase Reveals a “Pinch-Pull-Push” Mechanism*

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Uracil-DNA glycosylase catalyzes the excision of uracils from DNA via a mechanism where the uracil is extrahelically flipped out of the DNA helix into the enzyme active site. A conserved leucine is inserted into the DNA duplex space vacated by the uracil leading to the paradigmatic “push-pull” mechanism of nucleotide flipping. However, the order of these two steps during catalysis has not been conclusively established. We report a complete kinetic analysis of a single catalytic turnover using a hydrolyzable duplex oligodeoxyribonucleotide substrate containing a uracil-2-aminopurine base pair. Rapid chemical-quenched-flow methods defined the kinetics of excision at the active site during catalysis. Stopped-flow fluorometry monitoring the 2-aminopurine fluorescence defined the kinetics of uracil flipping. Parallel experiments detecting the protein fluorescence showed a slower Leu191 insertion step occurring after nucleotide flipping but before excision. The inserted Leu191 acts as a doorstop to prevent the return of the flipped-out uracil residue, thereby facilitating the capture of the uracil in the active site and does not play a direct role in “pushing” the uracil out of the DNA helix. The results define for the first time the proper sequence of events during a catalytic cycle and establish a “pull-push”, as opposed to a “push-pull”, mechanism for nucleotide flipping.

Escherichia coli uracil-DNA glycosylase (Ung) catalyzes the excision of uracils from DNA that arise either from the spontaneous deamination of cytosines or the incorporation of dUMP during DNA synthesis (1). The current model of the chemo-mechanical steps associated with uracil-DNA glycosylase activity is largely built around protein structural information derived from studies with the E. coli (2–4), the human (5–9), and the herpes simplex virus type-1 (2, 10, 11) uracil-DNA glycosylase. Central to the current understanding of the enzyme mechanism is the observation that the target uracil must be extracted from the DNA helix with the uracil-containing nucleotide rotated ~180° about the phosphodiester backbone to enter the active site (12). Co-crystal structures of the human enzyme bound to excision products show three distinctive structural features thought to be characteristic of the catalytic mechanism: 1) distortion of the DNA backbone imposed by a “pinching” action of a trio of serines, 2) displacement of the scissile uracil base from helical space of the DNA helix by the insertion of a conserved leucine side chain into the minor groove of the DNA, and 3) capture of the “flipped out” uracil by a binding pocket on the protein designed to stabilize the expelled uracil (5–7, 12). These observations have led to the proposal of a “pinch-push-pull” mechanism to describe the sequential steps associated with uracil capture in the active site. This paradigmatic model hypothesizes that the serine-pinches induces stress on the DNA structure, which is relieved when Leu191 of Ung is inserted to push the uracil out of the base stack in the DNA helix, thereby allowing it to be pulled into the active site.

Nucleotide flipping is not unique to the uracil-DNA glycosylase reaction mechanism of Ung (13–16) but was first observed in the crystal structure of the HhaI methylase (17). Although the reactions catalyzed by these diverse enzymes are clearly different, a common mechanism of nucleotide flipping has been proposed (13–16, 18). However, the pinch-push-pull reaction sequence for nucleotide flipping proposed for Ung appears inconsistent with structural data for the HhaI methylase. For example, O’Gara et al. (19) observed a “flipped-out” configuration of the deoxyribose-phosphate backbone in co-crystals of HhaI methylase bound to DNA despite replacement of the target cytosine residue of the recognition sequence with an abasic site. This observation shows clearly that nucleotide flipping can occur in the absence of a base to be pushed, suggesting that the ultimate driving force for the flipping must therefore be derived from DNA backbone distortion.

Because it is difficult to resolve a dynamic question of reaction sequence from static protein structures, direct kinetic measurement of the rates for the pushing and pulling steps of nucleotide flipping and leucine insertion must be obtained. Stivers et al. (20) reported such a study using stopped-flow fluorometry to investigate the kinetics of nucleotide flipping and leucine-insertion of the E. coli Ung during binding to an oligodeoxyribonucleotide containing a reporter fluorophore, 2-aminopurine, positioned adjacent to a non-cleaveable analog of uracil, 2′-fluoro-2′-deoxyuridine. With this substrate-analog, identical rates of nucleotide flipping and leucine insertion were observed that led to the conclusion that pushing and pulling must both occur simultaneously. However, Handa et al. (21) and Jiang and Stivers (22) recently reported kinetic parameters for the excision of uracils from a single-stranded DNA.
substrate, which showed a 15-fold reduction in $k_{\text{on}}/K_{D}$ between the wild-type and a L191A mutant E. coli Ung. If the role of the Leu insertion were to facilitate uracil flipping by pushing the scissile base out of the structural cage of the double helix, then it is difficult to rationalize why the L191A mutation should have such a pronounced effect on the excision of a uracil from a single-stranded DNA substrate where the steric impediments to flipping imposed by the duplex DNA structure are absent. These results suggest that the leucine insertion step likely plays a more complex role than that of a simple piston designed to dislodge a recalcitrant uracil base from the double helix.

In this report, we present the result from a combination of rapid chemical-quenched-flow and stopped-flow fluorometry studies that evaluated the pre-steady-state kinetics of the entire catalytic mechanism from binding through nucleotide flipping, leucine insertion, uracil excision, and product release using a uracil-containing duplex oligodeoxynucleotide substrate. A global, computer-simulated fit of all kinetic data to a single minimal reaction mechanism and a unique set of microscopic rate constants are presented. The structural data will be discussed in the context of the kinetic model to further elucidate the temporal and dynamic relationships between the static structures. Comparison of similarities and differences of our results to those of Stivers and coworkers (23) using the non-cleavable uracil analog will be discussed with regards to clarifying the role of leucine insertion in facilitating the catalysis of excision.

EXPERIMENTAL PROCEDURES

Materials and Buffers—T4 polynucleotide kinase was purchased from New England BioLabs (Beverly, MA) and [$\gamma$-32P]ATP (6000 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). All buffers were made from reagent grade chemicals. All kinetic experiments were carried out in buffer A containing 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 5% (w/v) glycerol. For stopped-flow experiments, spectrometric grade glycerol (EM Science) was used.

Enzymes—E. coli Ung was purified as Fraction V from JM105/pSB1051 as described by Sanderson and Mosbaugh (24). The purified Ung had a specific activity of 1.2 × 10^6 units per milligram of protein as determined by the release of [\text{3H}]uracil from activated calf thymus DNA (25). A final enzyme stock of 24.6 μM, as determined spectrophotometrically using ε_{290} = 4.22 × 10^4 M⁻¹ cm⁻¹, was stored until needed in buffer A at -80 °C.

Oligodeoxynucleotides—Synthetic oligodeoxynucleotides, U-27-mer (5'-GGGGCTGTCAGAACTGTCGTACC), A-27-mer (5'-CCCCGGAATTCTGATCAG), and 2AP-27-mer (5'-CCCCGATGACGAACTGTCGTACCAG) were purchased from Midland Certified Reagent Co. (Midland, TX). The concentrations of single-stranded oligodeoxynucleotides were determined spectrophotometrically using ε_{260} of 2.59 × 10^4 M⁻¹ cm⁻¹, 2.58 × 10^4 M⁻¹ cm⁻¹, and 2.49 × 10^4 M⁻¹ cm⁻¹ for U-27-mer, A-27-mer, and 2AP-27-mer, respectively. The U-27-mer oligodeoxynucleotide was 5'-32P-labeled as described previously by Bennett et al. (26). Duplex oligodeoxynucleotides, U-A-27-mer and U-2AP-27-mer, were hybridized at a 1:1.1 molar ratio with the A- or 2AP-containing oligodeoxynucleotide in slight excess in buffer containing 150 mM NaCl, 15 mM sodium citrate. Annealing was carried out by heating the samples to 70 °C for 5 min followed by slow cooling to room temperature. The extent of annealing (>95%) was ascertained by native gel electrophoresis in 12% polyacrylamide (26).

Steady-state Fluorescence Spectroscopy—Fluorescence spectra were obtained using a PerkinElmer Life Sciences LS50 luminescence spectrometer (excitation and emission slit widths of 10 nm, 5-mm path length, at 25 °C). Excitation spectra (250–350 nm) were collected by monitoring emission at λ_{em} = 370 nm whereas emission spectra (330–500 nm) were collected by excitation at λ_{ex} = 310 nm. Fluorescence titrations and time courses were performed at λ_{ex} = 310 nm and λ_{em} = 370 nm.

RAPID QUENCH ANALYSIS—Single-turnover and active site titration experiments were performed using a KinTek RFQ-3 Rapid Chemical Quench (KinTek Instruments, State College, PA) instrument maintained at a constant 25 °C with a Neslab RTE-111 refrigerated water bath. Reactions were initiated by rapidly mixing 15 μl of Ung with 15 μl of 5'-32P-UTP:2AP-27-mer substrate. Reactions were then quenched with 90 μl of quench solution containing 6 mM NaOH, 4 mM guanidine thiocyanate. Samples were immediately diluted 2-fold in distilled water, vortexed, and incubated at 55 °C for 1 h to cleave the sugar-phosphate backbone of the DNA at abasic sites. After cleavage, the samples were diluted 5-fold with water and analyzed using denaturing 12% polyacrylamide/8.3 M urea gel (26). 32P-labeled substrate and product oligodeoxynucleotide bands were visualized using a Molecular Dynamics PhosphorImager and quantitated using ImageQuant NT 5.0 (Molecular Dynamics). A t = 0 sample was obtained by mixing buffer instead of enzyme solution with substrate, and a t = ∞ point was obtained at 30 s to determine the maximum extent of attainable product. The concentration of excised uracil at time t, [P], was calculated according to Equation 1,

\[ [P]_t = [DNA]_0 \left( \frac{[IP]/([A]_0 + [IP])}{[IP]/([A]_0 + [IP])} \right) \]

where [DNA]_0 represents the initial concentration of DNA, $I$ denotes the intensities of bands, the subscripts $P$ and $S$ refer to product and substrate, respectively, and the subscripts, $t$, 0, and $\infty$, denote reaction times. Single-turnover time courses with enzyme present in excess over substrate were fitted to a single exponential according to Equation 2,

\[ [P]_t = A_1 (1 - e^{-k_{obs} t}) \]

while active-site titration experiments with excess substrate were fitted to a single exponential followed by a line according to Equation 3,

\[ [P]_t = A_1 (1 - e^{-k_{obs} t} + k_{obs} t) \]

where $A_1$ and $k_{obs}$ denote the amplitude and the apparent rate constant of the exponential phase and $k_{obs}$ denotes the linear steady-state rate constant. The dependence of $k_{obs}$ on [Ung] was fitted according to Equation 4,

\[ k_{obs} = k_{D} + ([E]_0 + [D]_0) \sqrt{k_{D} + ([E]_0 + [D]_0)^2 - 4([E]_0[D]_0)} \]

Steady-state Fluorescence Spectroscopy—Fluorescence spectra were measured using a Cary 4000 spectrophotometer equipped with a 100-W xenon arc lamp and a cooled photomultiplier tube. The emission was excited at 290 nm and the excitation spectra were scanned at 5-nm intervals from 250 to 350 nm. The fluorescence spectra were corrected for the absorbance at the excitation wavelength.

RESULTS

Fluorescence Properties of 2AP-27-mer and U-2AP-27-mer—The duplex U:2AP-27-mer consisted of a uracil-containing oligo-
godeoxyribonucleotide, U-27-mer, annealed to the complementary and fluorescent 2-aminopurine (2AP) containing 2AP-27-mer. The resulting duplex DNA contained a single uracil at position 18 from the 5’-end of U-27-mer that was positioned directly across from the 2AP base of the 2AP-27-mer. This substrate was designed to take advantage of the fluorescence properties of the 2AP base for detecting the uracil flipping reaction during catalysis. The fluorescence excitation and emission spectra of the single-stranded 2AP-27-mer (Fig. 1A) showed the characteristic excitation and emission maxima of 310 and 370 nm, respectively. The observed emission spectra (λ<sub>em</sub> = 310 nm) of a 400 nM solution of 2AP-27-mer showed linear, incremental loss of fluorescence upon addition of increasing concentrations of the complementary, uracil-containing oligodeoxyribonucleotide, U-27-mer up to an equimolar concentration of 400 nM as expected (Fig. 1B). Addition of excess U-27-mer apparently resulted in no further quenching. Spectra were collected 10 min following each addition of U-27-mer. Time courses of the observed fluorescence at the maximum emission wavelength of 370 nm after each addition of U-27-mer showed no further change in fluorescence after 10 min (Fig. 1C). The quantitative correlation between the observed fluorescence quenching of the 2AP-27-mer upon duplex formation is consistent with literature reports of 2AP as a sensitive probe for the single-stranded nature of its microenvironment within duplex DNA (20, 29–31) and confirms the usefulness of the resultant duplex, U:2AP-27-mer, as a stopped-flow probe for detecting uracil flipping.

**Rate of Uracil Excision at the Active Site of Ung**—To measure the rate of uracil excision at the enzyme active site, a single-turnover assay was performed in a rapid chemical quenched-flow apparatus using 2 μM 5'-32P-U:2AP-27-mer and 2 μM Ung. At various times up to 200 ms, a quench solution containing 6 M NaOH and 4 M guanidine thiocyanate was added. Enzymatically generated abasic sites within the oligodeoxyribonucleotide product were cleaved by incubation at 55 °C for 1 h, and the cleaved products were separated on a 12% polyacrylamide/8.3 M urea denaturing sequencing gel. Radioactive bands corresponding to the 32P-U-27-mer strand of the substrate and the 32P-17-mer product were visualized (Fig. 2A) and quantitated using a Molecular Dynamics PhosphorImager. Two secondary product bands were observed migrating slightly slower than the expected 17-mer product when alkaline cleavage was carried out in the presence of guanidine or urea while a single 32P-17-mer product band was observed when the reactions were quenched in 6 M NaOH alone. However, the guanidine thiocyanate was essential for efficient quenching of the reaction as control experiments showed that 6 M NaOH alone failed to quench the Ung-catalyzed reaction at times <10 ms (data not shown). These extra bands were therefore attributed to alternate chemical mechanisms of abasic site cleavage catalyzed by the guanidine. When the radioactivity contained in all product bands was summed to assess the total amount of uracil excised from the 32P-U:2AP-27-mer, a time-dependent conversion of the 27-mer to the 17-mer product was observed.

**Fig. 2. Single-turnover uracil excision catalyzed by Ung**. A, phosphorimaging profile of a 12% polyacrylamide/8.3 M urea gel showing the Ung-catalyzed time-dependent conversion of the 5'-32P-U:2AP-27-mer, S, to products, E. coli Ung (2 μM) was reacted with 2 μM 5'-32P-U:2AP-27-mer in a rapid quenched-flow apparatus and quenched at times shown as described under “Experimental Procedures.” The presence of guanidine from the quench solution during the alkaline cleavage step resulted in multiple cleavage products as indicated by the square bracket. B, the observed rate constants, k<sub>obs</sub>, for the excision of uracil from 10 nM 5'-32P-U:2AP-27-mer at different concentrations of Ung were plotted and fitted to Equation 4 to obtain best-fit parameters for the maximal single-turnover excision rate constant, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>, the observed rate constants, k<sub>B</sub>.
Ung, the amplitude of the exponential DNA concentrations increased with increasing concentration of DNA. At higher, 1.5, 2.5, 3.0 μM Ung (2 μM) was reacted with 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μM Ung (2 μM) 32P-U:2AP-27-mer in a KinTek RQF-3 rapid quenched-flow apparatus. The graph shows moles of excision products per mole of Ung added, Eo. Solid lines represent the non-linear least-squares best fit of data to Equations 2 or 3 as described under “Experimental Procedures.” B, the burst amplitudes obtained from fits in A were plotted as a function of 32P-U:2AP-27-mer concentration.

A = (Ko,app + aEo + |D|)/\sqrt{(Ko,app + aEo - 4aEo[D]/2Eo)^2},

where Eo = 2 μM, |D| represents the concentration of U:2AP-27-mer, Ko,app = 38 nM as determined from previous experiments, and a represents the fraction of the added Ung that was catalytically competent.

Active-site Titration and Burst Kinetics—To examine the relationship between the first catalytic turnover and the steady state, active site titration experiments were performed at 2 μM Ung with 0.5 μM (substoichiometric) to 3 μM (excess) 5′-32P-U:2AP-27-mer. The amount of product formed as a function of time at each concentration is shown in Fig. 3A. At substoichiometric substrate concentrations, the time courses were best fitted to a single exponential with a DNA concentration-independent rate constant, kobs, of 38 s⁻¹ and a corresponding dissociation constant, Ko, of 38 nM.

Active-site Titration of the Presteady-state Burst of Ung catalyzed uracil excision. A, Ung (2 μM) was reacted with 0.5 (●), 1.0 (○), 1.5 (■), 2.0 (□), 2.5 (▲), and 3.0 μM Ung (2 μM) 32P-U:2AP-27-mer in a KinTek RQF-3 rapid quenched-flow apparatus. The graph shows moles of excision products per mole of Ung added, Eo. Solid lines represent the non-linear least-squares best fit of data to Equations 2 or 3 as described under “Experimental Procedures.” B, the burst amplitudes obtained from fits in A were plotted as a function of 32P-U:2AP-27-mer concentration.

The pulse-trap experiment relied on the same approach with the exception that a solution of the PBS2 uracil-glycosylase inhibitor protein, Ugi, was used in place of the chase solution to selectively bind to free Ung and thereby prevent any re-binding of DNA substrates (25). The Ugi protein has been shown previously to inhibit Ung activity by forming an essentially irreversible 1:1 complex by acting as a DNA mimic and associating with the DNA binding site of Ung (4, 25, 37). In two parallel sets of reactions, Ung (2 μM) was reacted with 5′-32P-U:2AP-27-mer (1 μM) and terminated at various times either with a 30 μM Ugi “trap” solution or with the 6 mM NaOH/4 mM guanidine thiocyanate “quench” solution. Quench was added to the Ugi-trapped reactions after an additional 0.5 s to ensure adequate time for complete flux to occur. Following alkaline cleavage, denaturing polyacrylamide gel electrophoresis, imaging, and quantitation, the extent of cleaved products in each series of parallel reactions was plotted as shown in Fig. 4A. The “quenched” reaction showed the expected single-exponential time course with the expected kobs = 38 s⁻¹. However, the “trapped” reaction showed an excess of products in the earliest time points, which extrapolated to a y-intercept value comparable to 33% of the bound DNA.

Such an active-site titration plot (Fig. 3B) showed a linear increase with concentration up to a maximum plateau value of 0.88 upon addition of an equimolar amount of 2 μM DNA, indicating that 88% of the Ung preparation was catalytically active.

Pulse-trap Experiment—To investigate the internal dynamics of steps leading up to bond cleavage, a pulse-trap experiment was performed. This experiment is analogous to the pulse-chase experiment (33–36) in which a reaction time course obtained by adding a “chase” solution of excess unlabeled substrate at various times is compared with a parallel time course where a denaturing quench solution is added instead. Because the addition of the chase solution does not denature the enzyme but does prevent the detection of any additional DNA binding or re-binding, enzyme-bound substrates at the active site can proceed toward additional product formation provided it does so prior to dissociation from the active site as substrate. In contrast, the addition of a denaturing quench effectively precludes additional “flux.” The difference between the two parallel time courses, therefore, is a direct reflection of the relative probabilities of forward versus reverse flux for the bound substrate.

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Fig. 4B provides an illustration of the rationale behind the interpretation of these results in terms of the forward and reverse flux of an enzyme-bound substrate complex. In the quenched reaction, products appeared at the excision rate of 38 s⁻¹. With the addition of the Ugi trap solution at various time points, free Ung was sequestered and prevented from binding or re-binding substrate, but the DNA substrate-bound enzyme continued to be converted into products. Dynamic interchange of bound and free substrate pools were silent in the quenched assay but become apparent in the trap assay because only those bound substrate molecules that did not dissociate would be converted to additional product. Because the reaction conditions were chosen to ensure rapid equilibrium binding of DNA substrate as indicated by the maximal kobs of 38 s⁻¹, the Ugi solution should have been able to trap up to 100% of the bound DNA, if bound substrate did not dissociate prior to excision. The observed 33% trapping of bound DNA, therefore, reflects a net forward flux of 0.33 per binding event. Because forward flux was limited by the rate of excision at 38 s⁻¹, the net 33% probability of forward partitioning would dictate a 67% probability for the reverse, i.e. substrate dissociation. A net rate, koff of 77 s⁻¹ was determined. Experiments performed at higher
concentrations of 3 and 5 μM Ugi showed identical partitioning, indicating that Ugi did not interact directly with Ung-DNA complexes (data not shown). Control experiments where 30 μM Ugi was mixed with substrate prior to addition of the Ung solution showed no detectable product formation (data not shown), indicating that the 30 μM Ugi provided an efficient and rapid trap of free Ung.

**Stopped-flow Analysis of DNA Binding and Uracil Flipping**—To study the kinetics of the events leading up to excision, stopped-flow experiments were performed with U:2AP-27-mer (10 nM) and excess Ung (38–800 nM). The fluorescence emission of 2AP (Fig. 5A) showed a rapid, transient increase within the first 4–8 ms followed by a small but reproducible decrease. The amplitude of this second phase was too small to fit accurately and may be due to interference from protein fluorescence changes (see below). The initial rapid phase, however, yielded single-exponential best-fit rate constants in the range of 331 to 886 s⁻¹ and was hyperbolically dependent on Ung concentration. The non-linear dependence of the observed rate constant on concentration ruled out a direct one-step binding event coincident with uracil flipping.

**Stopped-flow Analysis of a Ung Conformation Change following Uracil Flipping**—The intrinsic tryptophan fluorescence of Ung (∆λex = 290 nm, Δλem > 320 nm) was monitored by stopped-flow to detect the transient conformation change of Ung in conjunction with the insertion of Leu⁹¹ into the DNA helix space vacated by the uracil. To eliminate interference from the 2AP fluorescence change, these experiments were performed at 10 nM Ung using the analogous non-fluorescent duplex U:A-27-mer (25–500 nM) in which the 2AP base was replaced by adenine. The kinetic trace obtained at each DNA concentration (Fig. 5B) was biphasic showing rapid and strong quenching within the first 20 ms followed by a much slower, partial recovery of the quenched fluorescence over several hundred milliseconds. Empirical exponential fit of the fast phase yielded hyperbolically dependent apparent rate constants that clearly reached a plateau at a value of ∼350 s⁻¹, which is smaller than the value of the rate constant for uracil flipping. Interestingly, the slow phase occurred with a maximal rate constant of 15 s⁻¹, a value that was smaller than the rate constant for cleavage.

Experiments conducted using U:2AP-27-mer were uninterpretable when emission was monitored using a cut-off filter due to interference from the 2AP fluorescence signal. However, traces obtained with λexc = 280 nm and a narrow band-pass filter at λem = 335 nm to minimize 2AP fluorescence showed tryptophan quenching at rates identical to those reported above for the non-fluorescent DNA substrate.

**Global Simulation Analysis of Stopped-flow Data**—Global analysis of all data traces were obtained using KinTekSim to a unified minimal kinetic scheme with a single set of rate constants as shown in Fig. 6 and Table I. Simulated stopped-flow traces using this model generated excellent fits to both 2AP and tryptophan fluorescence traces (Fig. 5A and B, solid lines). Global fits were obtained without adjustment of output factors between data sets. The model predicted a three-step binding mechanism prior to irreversible chemical bond cleavage: 1) rapid equilibrium binding of Ung with DNA complexes, E′S, during the trapping period, t₂. However, any E′S that does not dissociate during t₂ is converted to additional products in excess over those observed in the quenched reactions. The difference between the two time courses extrapolated to t₂ = 0 represents the fraction of E′S that partitioned in the forward direction to yield products as opposed to the fraction that dissociated as substrate.

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Fig. 6. Minimal kinetic scheme. The global fit was obtained using the minimal scheme shown. $E$, $D$, and $U$ denote Ung, DNA, and uracil, respectively. $E$ represents the initial conformation of the enzyme; $E_L$ represents the conformation with Leu$^{191}$ inserted into the DNA helix. $D_0$ and $D_L$ denote intra- versus extra-helical conformations of the uracil in the substrate DNA. Best-fit values of the microscopic rate constants are listed in Table 1.

**DISCUSSION**

**Mechanistic Implications of Kinetic Model**—This study represents the first complete kinetic analysis of the reaction pathway of *E. coli* uracil-DNA glycosylase using a duplex DNA substrate containing a fluorescent analog of the naturally occurring U:A lesion. By conducting the experiments under identical reaction conditions and using the same DNA substrate, we were able to obtain a unique, self-consistent set of microscopic kinetic constants, which quantitatively accounted for all of the observed data using a single, minimal kinetic scheme. This reaction scheme provides a novel model for explaining the Ung reaction mechanism and reveals several previously unidentified key features of the reaction mechanism as follows. 1) Substrate DNA is bound at least one order of magnitude faster than expected for a diffusion-control limited process thereby implicating some form of a facilitated diffusion search mechanism for locating U:A base pairs. 2) The extrahelical flipping of the uracil occurs faster than and therefore prior to the insertion of Leu$^{191}$ into the DNA helix. This insertion therefore functions not so much like a piston to “push” the uracil out of the DNA helix but more like a ratchet that “slides” into place to prevent the flipped-out uracil from slipping back into the DNA helix. 3) Uracil flipping occurs rapidly and reversibly. Return of the extracted uracil prior to the Leu$^{191}$ insertion occurs at 350 s$^{-1}$ with a net $K_{eq}$ for uracil capture by the binding pocket of 3.1. 4) In addition, irreversible $N$-glycosidic bond cleavage occurs at a macroscopic rate of 38 s$^{-1}$ following the Leu$^{191}$ insertion with a net product forming partitioning factor of 33%. 5) An Ung conformation change, which likely reflects the retraction of the inserted Leu$^{191}$, occurs after excision and prior to release of product at the steady-state turnover rate of 0.5 s$^{-1}$. Implications of these findings for various aspects of the mechanism of substrate recognition are discussed below.

**Global Fit to a Unique Reaction Scheme**—The proposed minimal kinetic scheme was supported by a unique, self-consistent set of elementarily rate constants obtained under a single set of reactions for a unique substrate. Previous reports in the literature have measured isolated segments of the reaction pathway using different substrates (20, 32, 38, 39). It is not possible to test the self-consistency of a global mechanism comprising the sum of segments derived from experiments using different substrates. Because global self-consistency remains the key test of any kinetic model (33, 34), the mechanistic relevance of disjointed segments of the pathway could not be previously ascertained. In addition, some of the segments, most notably those for the binding and uracil flipping steps leading up to excision, have been previously examined using substrate molecules that differed in substantial ways from the U:A containing duplex DNA.

**In Search of the U:A Lesion**—The hyperbolic, non-linear concentration dependence of the rate of 2AP fluorescence enhancement observed in the U:2AP-27-mer stopped-flow experiments revealed a rapid equilibrium step for the initial DNA binding reaction. The $K_{eq}$ of $3.6 \times 10^6$ M$^{-1}$ for this initial binding step was constrained by the concentration dependence of the observed rate constants and is within a factor of two from the $K_{eq}$ of $7.3 \times 10^6$ M$^{-1}$ reported by Stivers et al. (32) for an oligodeoxyribonucleotide containing the non-cleavable, 2’-β-fluoro-deoxyuridine analog. Although the rate of this step was too fast to be measured directly using any of the experimental techniques described, our minimum estimate of $k_1 \geq 1.8 \times 10^{10}$ M$^{-1}$s$^{-1}$, which exceeds the reported value of $5.5 \times 10^9$ M$^{-1}$s$^{-1}$, was based on two additional constraints that became apparent during the simulations. 1) It was necessary to maintain large values of $k_2$ and $k_3$ to account for the absence of a significant lag phase in the onset of the 2AP fluorescence enhancement. 2) Lowering $k_1$ to the diffusion-control limit of $1 \times 10^9$ M$^{-1}$s$^{-1}$, which was still 2-fold higher than the value used by Stivers et al. (20), required a corresponding 18-fold reduction of $k_1$, which made it virtually impossible to fit any of the 2AP stopped-flow traces obtained in the lower concentration range of Ung.

The observation that $k_1$ exceeded the diffusion-controlled limit of $1 \times 10^9$ M$^{-1}$s$^{-1}$ by 20-fold was consistent with facilitated diffusion (40-42). Although the 23-mer duplex DNA substrate contained only a single specific binding site as defined by the U:2AP base pair, it contains up to 22 additional nonspecific sites, depending on the minimum number of base pairs required by Ung to achieve stable DNA binding. Consequently, the concentration of total sites available for initial nonspecific binding could be as much as 23-fold higher than the concentration of the oligodeoxyribonucleotide substrate. Thus the apparent association rate constant would be expected to increase by a corresponding amount. Facilitated diffusion has been implicated in the targeted search mechanism of *E. coli* Ung (26, 43) and would provide a facile means of increasing scanning speed for specific recognition sites (44, 45). By taking advantage of the higher concentration of nonspecific sites available for initial binding and then searching for specific sites while remaining nonspecifically bound to the DNA lattice, the dimensionality of the search is reduced from 3 to 1 with concomitant increase in search efficiency (40-42).

**A Push versus Pull Mechanism**—In the case of the human uracil-DNA glycosylase, three structural perturbations have been reported in association with target recognition based on DNA co-crystal structures: the “serine-pincho” distortion of the DNA helix, the flipping out of the uracil, and the insertion of Leu$^{272}$ (homologous to Leu$^{191}$ in *E. coli* Ung) into the DNA helix (5, 6). The serine-pincho was proposed to occur on initial binding to nonspecific DNA (5) whereas the nucleotide-flipping and leucine-insertion events were purported to occur after specific recognition of uracil. Slupphaug et al. (6) hypothesized a direct “push and pull” mechanism whereby Leu$^{272}$ was envisioned as a piston that actively pushes the uracil from the DNA helix whereupon it becomes captured, or pulled in, by the active site of uracil-DNA glycosylase. However, it is not possible to deduce the sequential order of events from static protein structures alone. Although the proposed mechanism seems reasonable, it assumes that the uracil required exogenous impetus to exit the DNA helix. When a higher resolution structure of the enzyme-DNA complex was obtained, it revealed the serine-pincho helical distortion of the DNA backbone (5). Consequently, the original model was amended to describe a concerted pinch-push-pull mechanism where the order of events was not explicitly specified to accommodate the hypothesis that relief from the induced “helix strain” might provide the thermodynamic driving force to induce uracil flipping. The subsequent stopped-flow data of
Stivers et al. (20) provided the only previous evidence in support of a concerted mechanism where the pushing and pulling steps occur simultaneously. Unfortunately, these experiments were designed and performed using a non-cleavable substrate analogue (20).

The findings presented in this study represent the first direct evidence using a kinetically competent substrate to define the sequential order of DNA binding events. The results indicate that the uracil-flipping step precedes the leucine-insertion step. The evidence for this “pinch-pull-push” mechanism was derived from the two different spectroscopic signals observed in stopped-flow experiments. The first of these signals, which we ascribe to the uracil-flipping event, arose from the binding-induced fluorescence increase of a 2AP residue placed in the opposite strand of the DNA helix and base paired with the target uracil. The second spectroscopic signal, which we attribute to the leucine-insertion step, came from the quenching of the intrinsic tryptophan fluorescence of Ung and followed the 2AP fluorescence increase. The results unambiguously indicate that the event reported by the 2AP fluorescence signal occurred faster than and therefore prior to the event reported by the tryptophan fluorescence signal. However, the assignments of these two sequential events to the structural changes corresponding to the uracil-flipping and Leu-insertion step are somewhat more equivocal.

Several compelling lines of reasoning are offered to justify our assignments. With respect to the uracil-flipping signal, it is well established that the fluorescence quantum yield of 2AP is highly sensitive to the single-stranded nature of its environment (20, 29–31). This was also demonstrated here in the annealing titration experiment (Fig. 2). Therefore, it would be expected that the loss of base pairing due to nucleotide flipping would result in the enhancement of the 2AP probe’s fluorescence. In addition, the binding of an analogous duplex oligodeoxyribonucleotide containing thymine instead of the uracil failed to show any fluorescence increase under identical reaction conditions. This control experiment would corroborate the assignment of the 2AP signal to uracil flipping, because the thymine does not bind to the Ung active site. The assignment of the tryptophan signal to Leu insertion is somewhat more presumptive, because the fluorescence signal reflects a conformational change of the protein for which assignment to a specific structural perturbation is circumstantial. However, Stivers et al. (20) in describing stopped-flow data obtained using a non-cleavable substrate mimic, also observed a tryptophan-quenching signal that was assigned to the Leu insertion event based on the presence of two tryptophans, Trp$^{141}$ and Trp$^{164}$, near the Ung active site. In further support of this assignment, comparison of the human ung-DNA glycosylase structure obtained with and without DNA substrates revealed that leucine insertion into the DNA helix is accompanied by significant conformational changes surrounding these tryptophans. It would therefore not be surprising that such changes would influence their fluorescence properties. Because the observed stopped-flow tryptophan quenching signal is the only tryptophan fluorescence change detected prior to base excision, we, like Stivers et al. (20), propose that the observed quenching most likely reflects a conformational change associated with the leucine-insertion step.

Based on the assignments described above, it follows that nucleotide flipping must sequentially precede leucine insertion. A re-examination of the stopped-flow results as illustrated in Fig. 7 clearly establishes this point. The overlay of the stopped-flow traces of the 2AP and tryptophan signals obtained under comparable reaction conditions shows the prior occurrence of the 2AP fluorescence increase signifying uracil flipping. In contrast, the tryptophan quenching trace, signifying the insertion of Leu$^{191}$, lags behind the change in 2AP fluorescence. These results directly challenge any model that implies an active “pushing” role by the leucine in displacing the uracil from the DNA helix. To explain the data, we offer an alternate model that proposes the relatively facile and reversible flipping of the uracil between the uracil-binding pocket of Ung and the DNA helix. In addition, this new model suggests a ratchet-like action for Leu$^{191}$ in preventing the uracil from returning to the DNA helix.

Our results differ from those reported by Stivers et al. (20) whose stopped-flow data showed rate constants for uracil flipping of 1260 and 40 s$^{-1}$ in the forward and reverse directions, respectively. In addition, they observed identical rate constants for the 2AP and tryptophan fluorescence changes, leading them to conclude that the uracil flipping and leucine insertion occurred simultaneously. Although the two studies were carried out under somewhat different solution conditions, a more significant source of variation lies in differences between the DNA substrate used in the two studies. Whereas we used a DNA substrate containing a minimally perturbed (20, 29–31) U:2AP base pair as the target site, Stivers et al. (20) used a substrate mimicking the non-cleavable 2FU$^{5'}$G mispair. The observation of simultaneous nucleotide flipping and leucine insertion, therefore, may simply be a reflection of either the non-cleavable nature of the nucleotide analog or a property of the mismatched base pair. However, due to the slower rate of the bond-cleavage step relative to the rates for both nucleotide-flipping and leucine-insertion steps, the use of a non-cleavable analog to prevent excision was unnecessary.

**Inefficient Capture of Flipped Uracil**—The uracil-flipping step was observed to be freely and rapidly reversible with forward and reverse rate constants of 1100 and 350 s$^{-1}$, respectively, implying a surprisingly inefficient capture of the uracil by the enzyme’s active site with $K_{eq}$ for capture of 3.1. In addition, the pulse-trap experiment revealed that only 33% of the bound substrate was committed to a net forward flux resulting in excision. Together, these results suggest that both the nucleotide-flipping and the leucine-insertion steps merely provided the final impetus toward catalysis and that much of the proposed 10$^{12}$ catalytic efficiency (32) has already been achieved by the time the uracil is extruded. Hence, the majority of the driving force underlying catalysis is provided by the action of the serine-pinch. This seemingly contradicts the interpretations based on the crystallographic results that suggest an exquisitely tailored uracil-binding pocket molded to accommodate the uracil (3, 6). However, one must keep in mind that the helical space in the duplex DNA is also form-fitted to...

| n | Reaction                          | $k_a$       | $k_{-a}$     | $K_a$         |
|---|----------------------------------|-------------|--------------|---------------|
| 1 | Initial binding (nonspecific)    | $\geq 1.8 \times 10^{10}$ M$^{-1}$s$^{-1}$ | 5000 s$^{-1}$ | $3.6 \pm 0.3 \times 10^{9}$ M$^{-1}$ |
| 2 | Uracil flipping                  | 1.1000 ± 70 s$^{-1}$ | 350 ± 30s$^{-1}$ | 3.1           |
| 3 | Leu$^{191}$ insertion            | 300 ± 30 s$^{-1}$ | 170 ± 50s$^{-1}$ | 1.8           |
| 4 | Glycosidic bond cleavage         | 58 ± 4 s$^{-1}$ | 0            |               |
| 5 | Leu$^{191}$ retraction           | 35 ± 4 s$^{-1}$ | 25 ± 5 s$^{-1}$ | 1.5           |
| 6 | Steady-state $k_{cat}$ (product dissociation) | 0.5 ± 0.08 s$^{-1}$ |               |               |

"Pinch-Pull-Push" Mechanism of Nucleotide Flipping by Ung

### Table I

Rate and equilibrium constants.
accommodate the uracil, and that the $3.1 K_{eq}$ represents the difference in relative affinities for the binding of uracil in the two spaces. In this context, the uracil-binding pocket may be functioning less to capture the uracil to be excised as to prevent the capture of other, non-uracil bases that might be induced to flip into the binding pocket due to the helical strain induced by the action of the serine-pinch.

Additionally, although the uracil-binding pocket may be shaped to accommodate free uracil, it may not be optimized for binding a uracil base while attached via a glycosidic bond to the DNA duplex. Parikh et al. (7) observed that the enzyme prefers to bind a tetrahedral rather than the expected trigonal center of the uracil in the DNA duplex. This led them to hypothesize the “Bond Strain” model of catalysis in which application of physical strain at the C1 position “forces” the glycosidic bond into a more favorable alignment of electronic orbitals to facilitate catalysis (7). The poor efficiency of uracil capture we observed may reflect the “strain” involved in binding a uracil base still attached to the DNA via a glycosidic bond when the active site geometry is optimized to stabilize either the proposed uracil anion intermediate (38) formed during catalysis or the excised uracil product. In this context, however, the energetic driving force behind inducing the bond-strained complex would not come from the serine-pinch but from the leucine insertion, which prevents the uracil from slipping back into the DNA helix. In any case, the relief of strain imposed by the serine-pinch would be expected to persist at least through the transition state.

Rate of Uracil Excision—The 38 s$^{-1}$ rate of excision observed in the single-turnover experiments represents only a portion of the true microscopic rate constant, $k_{cat}$, of 58 s$^{-1}$. This is a consequence of the equilibrium constant of the preceding Leu insertion step. With a forward rate constant of 300 s$^{-1}$ and a reverse rate constant of 170 s$^{-1}$, only ~0.65 of the bound DNA molecules are conformationally poised to initiate the excision reaction. Therefore, the true microscopic rate constant for excision would be expected to exceed the observed rate constant by ~50%.

The 38 s$^{-1}$ rate constant for uracil excision observed was similar to but slower than the rate constant of 115 s$^{-1}$ reported by Drohat et al. (32) for a similar duplex DNA substrate. The apparent difference between these values did not arise from differences in activities between enzyme preparations, because the rate constants in question were obtained under saturating single-turnover conditions and were insensitive to enzyme concentration. Furthermore, the active-site titration experiments reported here indicated that the Ung preparation, with the slower 38 s$^{-1}$ excision rate constant, is 88% active. Instead, differences in buffer conditions, most notably the use of pH 7.4 buffer by us versus that of 8.0 in their experiments (32), may provide the most likely explanation for this observation.

Post Uracil Excision Events—The partial recovery of the tryptophan fluorescence quenching provided evidence of additional steps during catalysis. In particular, the rate constant for the recovery of the signal was smaller than the observed uracil excision rate constant of 38 s$^{-1}$ thereby placing the corresponding event after the chemical bond cleavage step. On the other hand, it was greater than the steady-state turnover number, $k_{cat}$, of 0.5 s$^{-1}$, which corresponds to the release of product DNA placing this conformation change step somewhere between uracil excision and DNA release. The fact that this step is accompanied by a recovery of the fluorescence lost during leucine insertion is consistent with it representing the reversal of leucine-insertion step.

The rate constants for this step in both directions are small, relative to all of the pre-excision rate constants, suggesting that catalysis was accompanied by a reduction in the overall dynamic motion in the active site. Consequently, we propose that the initial serine-pinch created a structural distortion where the uracil became unstably bound in the helix but that the active-site pocket may be unable to efficiently capture the flipped-out uracil because the glycosidic bond remains unbroken. Complete capture of the uracil for excision would require the helical insertion of the Leu$^{191}$ into the DNA to prevent the uracil from slipping back into the DNA helix. The large forward and reverse rate constants for the flipping and insertion steps support the hypothesis that the enzyme active site is not optimized to accommodate any one of the substrate-bound ground state structures, thereby providing the requisite preferential ground state destabilization to drive catalysis. In this model, the order of magnitude (300 to 25 s$^{-1}$ for leucine insertion) reduction of dynamic motion following excision would reflect the relief of strain-induced destabilization.

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Presteady-state Analysis of a Single Catalytic Turnover by *Escherichia coli* Uracil-DNA Glycosylase Reveals a "Pinch- Pull-Push" Mechanism

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