Coordinating the Initial Steps of Base Excision Repair

APURINIC/APYRIMIDINIC ENDONUCLEASE 1 ACTIVELY STIMULATES THYMINE DNA GLYCOSYLASE BY DISRUPTING THE PRODUCT COMPLEX

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DNA glycosylases initiate base excision repair by removing damaged or mismatched bases, producing apurinic/apyrimidinic (AP) DNA. For many glycosylases, the AP-DNA remains tightly bound, impeding enzymatic turnover. A prominent example is thymine DNA glycosylase (TDG), which removes T from G-T mispairs and recognizes other lesions, with specificity for damage at CpG dinucleotides. TDG turnover is very slow; its activity appears to reach a plateau as the product/ enzyme ratio approaches unity. The follow-on base excision repair enzyme, AP endonuclease 1 (APE1), stimulates the turnover of TDG and other glycosylases, involving a mechanism that remains largely unknown. We examined the catalytic activity of human TDG (hTDG), alone and with human APE1 (hAPE1), using pre-steady-state kinetics and a coupled-enzyme (hTDG- hAPE1) fluorescence assay. hTDG turnover is exceedingly slow for G-T (k_{cat} = 0.00034 min^{-1}) and G-U (k_{cat} = 0.005 min^{-1}) substrates, much slower than k_{max} from single turnover experiments, confirming that AP-DNA release is rate-limiting. We find unexpectedly large differences in k_{cat} for G-T, G-U, and G-FU substrates, indicating the excited base remains trapped in the product complex by AP-DNA. hAPE1 increases hTDG turnover by 42- and 26-fold for G-T and G-U substrates, the first quantitative measure of the effect of hAPE1. hAPE1 stimulates hTDG by disrupting the product complex rather than merely depleting (endonucleolytically) the AP-DNA. The enhancement is greater for hTDG catalytic core (residues 111–308 of 410), indicating the N- and C-terminal domains are dispensable for stimulatory interactions with hAPE1. Potential mechanisms for hAPE1 disruption of the of hTDG product complex are discussed.

The nucleobases in DNA are continuously modified by processes involving deamination, methylation, and oxidation, generating mutagenic and cytotoxic lesions that are implicated in aging and diseases including cancer and neurodegeneration (1, 2). Such damage is handled by the highly conserved base excision repair (BER) pathway, initiated by a damage-specific DNA glycosylase (3, 4). These remarkable enzymes use a nucleotide-flipping mechanism to find lesions and cleave the N-glycosidic bond, releasing the damaged base and producing an abasic or apurinic/apyrimidinic (AP) site in the DNA (5). Many DNA glycosylases remain tightly bound to their AP-DNA product, impeding enzymatic turnover. This may reflect a need to protect against the mutagenic and cytotoxic properties of AP sites, which impede some DNA polymerases, lack base coding information if replicated, and lead to single-strand breaks. Previous studies of BER in organisms ranging from Escherichia coli to humans have shown that AP endonucleases stimulate the activity (turnover) of many DNA glycosylases (6–10). These important findings suggest some degree of coordination in the initial steps of BER, yet the mechanism remains largely unknown.

We address this question here for human thymine DNA glycosylase (hTDG), which removes T from G-T mispairs and excises many additional lesions, with specificity for damaged bases that are paired with guanine and located in a CpG sequence context (11–15). A crystal structure of the hTDG catalytic domain (hTDG_{cat}, residues 111–308) bound to abasic DNA indicates the CpG specificity arises from interactions that select for guanine as the pairing partner of the target base and for guanine 3’ to the target base (i.e. 5’-CpG-3’/5’-XpG-3’, where X is the target base) (16). The specificity of hTDG for damage in a CpG context suggests the predominant biological substrate is G-T mispairs arising from deamination of 5-methylcytosine (m^{5}C) to thymine (17), because DNA methylation occurs at cytosine (C5) of CpG dinucleotides. CpG methylation is an epigenetic modification that plays a central role in regulating gene expression and maintaining genomic stability. Another human DNA glycosylase exhibits specificity for G-T mispairs at CpG sites, methyl binding domain IV (18–21), which may reflect a biological imperative to preserve the integrity of CpG sites. Nevertheless, CpG sites exhibit a disproportionately high frequency of mutations (C → T) in human cancers and genetic disease (22–24), and it was suggested this may be attributable in part to the slow turnover of hTDG (12). The

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3 The abbreviations used are: BER, base excision repair; AP, apurinic/apyrimidinic; FAM, 6-carboxyfluorescein; FU, 5-fluorouracil; HPLC, high pressure liquid chromatography; hTDG, human thymine DNA glycosylase; hTDG_{cat}, residues 111–308 of hTDG; m^{5}C, 5-methylcytosine; hUNG, human uracil glycosylase; hAPE1, human AP endonuclease 1; SUMO, small ubiquitin-like modifier.

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importance of understanding hTDG-initiated BER is underscored by findings that it may participate in the active demethylation of m⁵CpG sites, hence transcriptional regulation, by processing G-T mispairs created by active deamination of m⁵C to T (25, 26). Such a role is consistent with a preliminary report that homozygous knock-out of the TDG gene is embryonic-lethal in mice (13). We note that if demethylation of m⁵CpG sites involves active deamination, it would dramatically increase the burden of G-T mispairs, providing an alternative explanation for the high mutational frequency observed at CpG sites.

Previous studies have shown that hTDG exhibits exceedingly slow turnover after converting a stoichiometric amount of G-T or G-U substrate to G-AP product (7, 27, 28). This is attributable to very slow product release; a dissociation constant of \( k_{\text{off}} = -0.0016 \text{ min}^{-1}\) (half-life of 7 h) was estimated for G-AP-DNA on the basis of electrophoretic mobility shift studies (7). Accordingly, hTDG binds abasic DNA with high affinity (29), and abasic DNA is a potent inhibitor of hTDG (27). In contrast, hTDG is not inhibited by the nucleobases that it removes from DNA, including thymine or 3,4-ethenocytosine (εC), even at concentrations of up to 5 mM (17), indicating they do not bind hTDG with significant affinity.

It has been shown that human AP endonuclease 1 (hAPE1, also known as Ref-1) stimulates the turnover of hTDG for G-T, G-U, and GεCs substrates (7, 17, 30). However, the magnitude of the hAPE1 effect is unknown because the steady-state turnover \( k_{\text{cat}} \) of hTDG in the presence and absence of hAPE1 has not been reported for any substrate. Moreover, the mechanism of hAPE1 stimulation has remained elusive, although two basic ideas have been proposed. It has been suggested that hAPE1 stimulates hTDG by depleting (endonucleolytically) the concentration of AP-DNA, involving no interaction between hAPE1 and the hTDG product complex (13, 31), a mechanism that we will refer to as “passive enhancement.” On the other hand, the “active displacement” mechanism requires that hAPE1 interacts with hTDG and/or AP-DNA to disrupt the product complex (6, 7, 17, 30, 32) and may also involve hAPE1-mediated depletion of AP-DNA.

Here, we explore the mechanism by which hAPE1 stimulates hTDG turnover using an experimental approach that has not previously been employed to address this question in BER. Using pre-steady-state multiple-turnover kinetics, we determined the maximal turnover rate \( k_{\text{cat}} \) for hTDG acting upon G-T, G-U, and G-FU substrates. We developed a coupled-enzyme (hTDG-hAPE1) fluorescence assay to measure the steady-state kinetic parameters of hTDG in the presence of hAPE1. Together, these methods provide a quantitative measure of the hAPE1 effect. We also examined the stimulatory effect of hAPE1 on the turnover of hTDGcat, which lacks the N- and C-terminal domains of hTDG (110 and 102 residues, respectively). Our results rule out the passive enhancement mechanism and require that hAPE1 actively displaces AP-DNA from the hTDG product complex. The coupled-enzyme assay described here provides a new approach for studying the mechanism of hTDG using steady-state kinetics.

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**G·U20**

5′ DabT−TGCTCAUGTACAGAGCTGC
3′ FAMT−ACGAGTGCATGTCTCGACG

**G·T20**

5′ DabT−TGCTCATTACAGAGCTGC
3′ FAMT−ACGAGTGCATGTCTCGACG

**G·FU20**

5′ DabT−TGCTCAFGUTACAGAGCTGC
3′ FAMT−ACGAGTG_CATGTCTCGACG

**FIGURE 1.** DNA substrates used in this work. \( U \) represents 2′-deoxyuridine (dU), \( F \) represents 5-fluoro-dU, DabT and FAMT represent dabcyl-dT and 6-carboxyfluorescein-dT, respectively, where dabcyl and FAM are conjugated to the thymine base (C5 carbon) of dT (see Fig. 3). For each substrate the target base (bold) is located in a CpG dinucleotide context (underlined).

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA oligonucleotides were synthesized at the Keck Foundation Biotechnology Resource Laboratory of Yale University, purified by HPLC or Glen-Pack purification cartridges (Glen Research), and quantified by absorbance (260 nm) as described (14). Purity was verified by analytical anion-exchange HPLC under denaturing (pH 12) conditions (14). Phosphoramidites for special nucleotides were obtained from Glen Research. The duplex DNA substrates (Fig. 1) were hybridized by rapid heating to 80 °C followed by slow cooling to room temperature.

hTDG and the hTDG catalytic core (hTDGcat, residues 111–308 of 410) were expressed and purified as previously described (15, 16), quantified by absorbance \( (\epsilon_{280} = 17.7 \text{ mm}^{-1}\text{cm}^{-1}) \) for hTDGcat, \( \epsilon_{280} = 31.5 \text{ mm}^{-1}\text{cm}^{-1} \) for hTDG, flash-frozen, and stored at −80 °C.

hAPE1 was expressed in BL21(DE3) cells (Novagen) transformed with a pET-28 expression plasmid (generously provided by Prof. Ian Hickson, University of Oxford). Cells were grown at 37 °C to an \( A_{600} = -0.7 \), and hAPE1 expression was induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside for 4 h. hAPE1 was purified (at 4 °C) using essentially the same protocol as previously described for hTDGcat (17), including nickel-affinity chromatography (Qiagen), thrombin cleavage (overnight, 4°C) of the N-terminal polyhistidine tag (leaving six non-native N-terminal residues, GSHMAS), and anion exchange and size exclusion chromatography using SP-Sepharose and Superdex 75 columns (GE Healthcare). The molecular weight was confirmed by mass spectrometry. hAPE1 was quantified by absorbance \( (\epsilon_{280} = 52.9 \text{ mm}^{-1}\text{cm}^{-1}) \), flash-frozen, and stored −80 °C.

**Pre-steady-state Kinetics**—Transient kinetics experiments for hTDG (and hTDGcat) were conducted at room temperature (−22 °C) in HEMN.1 buffer (20 mM HEPES pH 7.5, 0.1 mM NaCl, 0.2 mM EDTA, 2.5 mM MgCl₂) with 0.1 mg/ml bovine serum albumin, quenched with 50% (v/v) 0.3 mM NaOH, 0.03 mM EDTA, and heated for 15 min at 85 °C to induce cleavage of the DNA backbone at abasic sites. Reactions were performed manually or by using a three-syringe rapid chemical quenched-flow instrument (RQF-3, Kintek Corp.). The extent of product formation was analyzed using an HPLC assay as described (14, 15).
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Single turnover turnovers were collected for hTDG (and hTDGcat) under saturating enzyme conditions ([E] >> [S] >> \(K_d\)) to obtain rate constants (for the maximal rate of AP product formation) that are not impacted by product release or the association of enzyme and substrate (14, 15). Data were fitted by non-linear regression to Equation 1 using Grafit 5 (33),

\[
[\text{Product}] = A\{1 - \exp(-k_{\text{obs}}t)\} + vt \quad \text{(Eq. 1)}
\]

where \(A\) is the amplitude, \(k_{\text{obs}}\) is the rate constant, and \(t\) is the reaction time (min). We use a concentration of enzyme, 5 \(\mu\)M, that is >100-fold higher than previously reported \(K_d\) values for hTDG (17, 34) and 0.5 \(\mu\)M substrate. These concentrations are sufficiently high to ensure saturating enzyme conditions, thereby providing the maximal rate constant for (enzyme-bound) product formation (i.e. \(k_{\text{obs}} \approx k_{\text{max}}\)), as shown in our previous studies (14, 15). To confirm saturating enzyme conditions, single turnover experiments were in some cases repeated with higher (10 \(\mu\)M) and lower (2.5 \(\mu\)M) enzyme concentrations, yielding the same result (within experimental error).

The maximal rate constant for enzymatic turnover (\(k_{\text{cat}}\)) was determined using pre-steady-state multiple-turnover kinetics conducted with a high enzyme concentration and excess substrate ([S] >> [E] >> \(K_d\)) such that \(k_{\text{cat}}\) was not limited by the association of enzyme and substrate (35). Progress curves exhibited “burst” kinetics, with a rapid exponential phase followed by a slow linear phase, indicating that the rate of product formation (enzyme-bound) greatly exceeds that of product release (35). Data were fitted to Equation 2,

\[
[\text{Product}] = A\{1 - \exp(-k_{\text{obs}}t)\} + vt \quad \text{(Eq. 2)}
\]

where \(A\) and \(k_{\text{obs}}\) are the amplitude and rate constant of the exponential phase, \(v\) is the steady-state velocity, and \(t\) is the reaction time (min). The steady-state rate constant (\(k_{\text{cat}}\)) was obtained by dividing the steady-state velocity (\(v\)) by the amplitude (A). In all cases, \(k_{\text{obs}}/k_{\text{cat}} \approx 150\), indicating the rate-limiting step occurs after chemistry. We used a high enzyme concentration (500 nM) and excess substrate (\(\approx 1 \mu\)M) to obtain maximal \(k_{\text{cat}}\) values (35), as confirmed by repeating experiments at lower enzyme concentrations.

Coupled-enzyme Fluorescence Assay—We developed a coupled-enzyme (hTDG-hAPE1) fluorescence assay to accurately determine the steady-state kinetic parameters of hTDG (\(k_{\text{cat}}\) and \(K_m\)) in the presence of hAPE1 (Fig. 2A). As is typical for a coupled-enzyme assay (36), the product of the primary enzyme (hTDG) is a substrate for the coupling enzyme (hAPE1), and the steady-state velocity of hTDG is monitored by the formation of hAPE1 product. As shown below and as expected for a coupled-enzyme assay (36), the initial velocity (\(v_0\)) directly reflects the steady-state turnover of hTDG because we used a sufficiently high concentration of hAPE1 to ensure that hTDG turnover was rate-limiting. It is important to note that the steady-state turnover of hAPE1 (alone) is at least 10,000-fold faster than that observed here for hTDG (alone) (37). Using the coupled-enzyme assay, the steady-state kinetic parameters for hTDG (\(k_{\text{cat}}\) and \(K_m\)) were obtained by determining the initial velocity (\(v_0\)) as a function of substrate concentration and fitting the data to the Michaelis-Menten equation (Equation 3) using non-linear regression with Grafit 5 (33),

\[
k_{\text{obs}} = k_{\text{cat}}[S]/(K_m + [S]) \quad \text{(Eq. 3)}
\]

where \(k_{\text{obs}} = v_0/[E]_{\text{tot}},\) and \([E]_{\text{tot}}\) is the total concentration of hTDG (or hTDGcat). The kinetic parameters \(k_{\text{cat}}\) and \(K_m\) directly reflect the steady-state activity of hTDG because the experiments (\(v_0\) determinations) were collected in the presence of sufficient hAPE1 such that the hTDG reaction was rate-limiting and \(v_0\) was independent of [hAPE1], as shown below.

The DNA substrates (Fig. 1) contain a fluorescence quencher (dabcyl) at the 5’ end of the substrate (i.e. dU-containing) strand and a fluorophore (FAM) at the 3’ end of the complementary strand, such that FAM fluorescence is initially quenched (Fig. 2A). The substrates are similar to those used for previous “molecular beacon” assays for hAPE1 (38, 39) but differ in one important respect as described below. The sequential activity of hTDG (creates an AP site) and hAPE1 (nicks the DNA backbone 5’ to the AP site) releases a short dabcyl-con-
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The individual reactions (i.e. $v_0$ determinations) were initiated by adding hTDG to HEMN.1 buffer containing the DNA substrate and hAPE1 and were monitored by the change in fluorescence intensity. A typical progress curve obtained from the coupled-enzyme assay is shown in Fig. 2C. As expected, hAPE1 alone does not generate a fluorescence change for the hTDG substrates, which do not contain an AP site (Fig. 2C, red data). The initial velocities ($v_0$) were obtained by linear regression of fluorescence intensity (cps) versus time (s) for $\sim 50$ s at the beginning of the progress curve, which is highly linear (Fig. 2C, inset). The $v_0$ values were converted from units of fluorescence (cps s$^{-1}$) to product concentration (nmol s$^{-1}$) using a conversion factor obtained from a plot of total fluorescence change ($\Delta F_{\text{tot}}$) versus substrate concentration. These standard curves ($\Delta F_{\text{tot}}$ versus [S]) were determined for [S] $\leq$ 500 nm and were quite linear ($r > 0.99$). Values for $\Delta F_{\text{tot}}$ were determined by bringing the coupled reaction to full completion. For the G-U20 and G-FU20 substrates, reactions were rapidly brought to completion by adding 25 nm human uracil DNA glycosylase (hUNG) after measuring $v_0$ for hTDG. The fluorescence cells were carefully cleaned after each experiment to thoroughly remove hUNG. This was verified by ensuring the fluorescence of substrate with hAPE1 did not change over time ($\sim 5$ min) before adding hTDG. The presence of residual hUNG would be clearly indicated by a fluorescence increase due to the sequential activity of hUNG and hAPE1.

Control experiments were routinely performed to confirm that the hAPE1 concentration was sufficiently high such that $v_0$ is independent of [hAPE1] and reflects the steady-state turnover of hTDG, i.e. that hTDG turnover is rate-limiting. For example, a series of $v_0$ determinations collected with 50 nm (dabcyl) were linked by phosphodiester bond to the 5’- or 3’-terminal oxygen of the DNA (Fig. 3). We prepared a G-U20 substrate using this approach and found that hAPE1 alone generates a rapid fluorescence increase even though the DNA contained no AP site. In contrast, hAPE1 did not change the fluorescence of a G-U duplex with FAM and dabcyl linked to the thymine base of a terminal dT nucleotide, which involves no phosphodiester bond (Fig. 3). Control experiments with four (non-AP) duplexes in which one group (either FAM or dabcyl) was linked (phosphodiester bond) to the 3’- or 5’-terminal oxygen and the other group was dT-linked revealed a fluorescence increase for hAPE1 alone in all cases (i.e. for 3’- or 5’-linked FAM and for 3’- or 5’-linked dabcyl). We conclude that hAPE1 removes FAM or dabcyl linked to either the 3’- or 5’-terminal oxygen of DNA, presumably by hydrolyzing the phosphodiester bond. This activity is significant, up to 13% of the AP endonuclease activity (not shown). Accordingly, FAM-dT and dabcyl-dT were used for our coupled assay (Fig. 1).

RESULTS

Kinetics of hTDG Alone—To advance our understanding of how hAPE1 stimulates the turnover of hTDG, it was necessary to first determine the kinetic parameters for hTDG in the absence of hAPE1. Fig. 4 shows a minimal kinetic mechanism for the hTDG reaction. After DNA binding, nucleotide flipping brings the target base into the hTDG active site, and cleavage of the N-glycosidic bond (and addition of the water nucleophile) gives the ternary enzyme-product complex. Two possible pathways for product release are shown, where AP-DNA can be released before or after the excised base. The kinetic parameter $k_{\text{max}}$ reflects the maximal rate of (enzyme-bound) product formation and is governed by the reaction steps after DNA binding and before product release (i.e. nucleotide flipping and base excision). The kinetic parameter $k_{\text{cat}}$ reflects the maximal steady-state turnover of hTDG and reflects the same steps as $k_{\text{max}}$ plus product release (Fig. 4). Although $k_{\text{max}}$ is readily

FIGURE 3. FAM and dabcyl labeling of DNA. A, for the DNA substrates used here, the dabcyl (quencher) and FAM (fluorophore) are linked to the thymine base (C5) of a terminal dT nucleotide. B, the previously described molecular beacon assays for hAPE1 used DNA in which dabcyl and FAM are linked by phosphodiester bond to the 3’- or 5’-terminal oxygen. Our control experiments indicate hAPE1 can remove dabcyl or FAM linked to either the 3’- or 5’-terminal oxygen (see “Experimental Procedures”).

hTDG$^{\text{cat}}$, 1500 nm G-U20 substrate, and varying hAPE1 concentrations (Fig. 2D) shows that $v_0$ is independent of hAPE1 concentration for [hAPE1] $\geq$ 50 nm. As expected, $v_0$ increases linearly with hTDG$^{\text{cat}}$ concentration (for 12–150 nm hTDG$^{\text{cat}}$) in the presence of a fixed (50 nm) concentration of hAPE1 (and 1500 nm substrate). A similar approach was used for G-T and G-FU substrates to ensure the $v_0$ values are independent of [hAPE1] and reflect the steady-state turnover of hTDG.

hAPE1 Removes FAM and Dabcyl Linked to the 5’- or 3’ Terminal Oxygen of DNA—Our coupled enzyme assay differs from previous molecular beacon assays involving hAPE1 (38, 39), for which the fluorophore (FAM) and quencher...
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**Kinetic Mechanism for hTDG.** The minimal kinetic mechanism is shown, including the steps that contribute to $k_{\text{max}}$, which reflects the maximal rate of product formation, and $k_{\text{cat}}$, which reflects the maximal steady-state turnover rate. The association of enzyme and DNA gives the collision complex (E-D), and nucleotide flipping gives the reactive enzyme-substrate complex (E-D'). The chemical step ($k_{\text{chem}}$) involves cleavage of the base-sugar (N-glycosidic) bond and the addition of water (nucleophile) giving the product complex (E-B-apD); where B is the excised base, apD is AP-DNA. Two potential pathways for product release are shown. Our finding that the excised base influences the dissociation rate of AP-DNA suggests AP-DNA is released before the base (upper pathway).

**FIGURE 4.** Kinetic mechanism for hTDG. Representative data from single turnover kinetics experiments collected for hTDG (500 nM) and G-U20 (1000 nM). After establishing the baseline kinetic parameters $k_{\text{obs}}$ and $k_{\text{cat}}$, we were able to calculate $k_{\text{max}}$ and $k_{\text{cat}}$. The results are essentially identical to G-U20, indicating that our conditions provide experimental error (within experiment error) were obtained for a lower hTDG concentration of 100 nM (not shown), indicating that our conditions provide the maximal turnover rate for hTDG (alone). We used single turnover kinetics to more accurately determine the maximal rate of (enzyme-bound) AP-DNA product formation, obtaining $k_{\text{max}} = 2.2 \pm 0.3 \text{ min}^{-1}$ (Fig. 5B). This is consistent with the exponential phase of the burst kinetics experiment (above) and all of our previous results from single turnover experiments for similar G-U substrates (14–16, 43). As a control, the kinetics experiments were repeated with the G-U19 substrate, which is identical to G-U20 but does not contain the terminal FAM-dT or dabcyl-dT groups (which are needed for the coupled-enzyme fluorescence assay, discussed below). The results are essentially the same, $k_{\text{max}} = 2.6 \pm 0.3 \text{ min}^{-1}$ and $k_{\text{cat}} = 0.007 \pm 0.001 \text{ min}^{-1}$ (not shown), indicating FAM-dT and dabcyl-dT do not perturb hTDG activity.

We also collected the burst kinetics and single turnover experiments for a G-T substrate, finding the steady-state turnover is exceedingly slow, $k_{\text{cat}} = 0.00034 \pm 0.00007 \text{ min}^{-1}$ (Fig. 5C), much slower than the maximal rate of product formation, $k_{\text{max}} = 0.09 \pm 0.01 \text{ min}^{-1}$ (Fig. 5D). The observation that $k_{\text{max}}$ exceeds $k_{\text{cat}}$ by 440- and 270-fold for G-U and G-T substrates, respectively, demonstrates that the turnover of hTDG ($k_{\text{cat}}$) is limited by a step after chemistry, likely AP-DNA product release (i.e. $k_{\text{cat}} \approx k_{\text{off}}$).

**Kinetics for hTDG Catalytic Domain**—We were also interested in examining the potential for hAPE1 to stimulate the turnover of hTDG$^{\text{cat}}$, which contains the region of high similarity (32% identical) to E. coli mismatch-specific uracil glycosylase (16, 28, 44, 45). Previous studies (28, 43) and our findings here (Table 1) show that hTDG$^{\text{cat}}$ has nearly the same catalytic activity as hTDG for most substrates even though it lacks the N- and C-terminal domains (110 and 102 residues, respectively). We find the steady-state turnover of hTDG$^{\text{cat}}$ (500 nM) for the G-U substrate (1000 nM) is slow, $k_{\text{cat}} = 0.006 \pm 0.002 \text{ min}^{-1}$ (Table 1), and is identical to that observed for full-length hTDG. Identical $k_{\text{cat}}$ values (within error) were obtained for a lower hTDG$^{\text{cat}}$ concentration (100 nM, not shown). Using single turnover experiments, we find the maximal rate of AP product formation for hTDG$^{\text{cat}}$ is much (150-fold) faster, $k_{\text{max}} = 0.9 \pm 0.1 \text{ min}^{-1}$ for the same G-U20 substrate (not shown). Control experiments using the G-U substrate which lacks the FAM and dabcyl groups (G-U19) gave essentially the same result, $k_{\text{max}} = 1.2 \pm 0.1 \text{ min}^{-1}$ and $k_{\text{cat}} = 0.005 \pm 0.001 \text{ min}^{-1}$, indicating that hTDG$^{\text{cat}}$ activity is not altered by FAM-dT or dabcyl-dT. These experiments were not collected for G-T20 because the G-T activity ($k_{\text{max}}$) was significantly lower for hTDG$^{\text{cat}}$ relative to hTDG.

**hAPE1 Does Not Affect hTDG Reaction Steps before Product Release**—Having established the baseline kinetic parameters $k_{\text{max}}$ and $k_{\text{cat}}$ for hTDG alone, we sought to establish which step(s) of the hTDG reaction is enhanced by hAPE1 and to quantify the magnitude of the effect. Previous studies suggested that hAPE1 stimulates hTDG product release (7, 17), but it was not established whether hAPE1 may also influence earlier steps of the hTDG reaction (i.e. nucleotide flipping or chemistry). To directly address this question, we repeated the single turnover experiments for hTDG and the G-U20 substrate in the presence...
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TABLE 1

Kinetic parameters for hTDG

| Substrate | Enzyme     | \(k_{\text{max}}^{\text{a}}\) | \(k_{\text{cat}}^{\text{a}}\) | \(k_{\text{max}}/k_{\text{cat}}\) | \(k_{\text{cat}}^{\text{APE1}}/k_{\text{cat}}\) | hAPE1 effect \(k_{\text{cat}}^{\text{APE1}}/k_{\text{cat}}\) |
|-----------|------------|-----------------------------|-----------------------------|-----------------------------|--------------------------------|-----------------------------|
| G-U20     | hTDG       | 2.2 ± 0.3                   | 0.005 ± 0.001               | 440                         | 0.133 ± 0.006                  | 26                          |
| G-U20     | hTDG\textsuperscript{cat} | 0.9 ± 0.1                   | 0.006 ± 0.002               | 150                         | 0.46 ± 0.02                    | 77                          |
| G-T20     | hTDG       | 0.092 ± 0.013               | 0.00034 ± 0.00007           | 272                         | 0.014 ± 0.001                  | 42                          |
| G-FU20    | hTDG       | 278 ± 35                    | 0.63 ± 0.17                 | 440                         | 0.54 ± 0.06                    | 0.9                         |
| G-FU20    | hTDG\textsuperscript{cat} | 111 ± 14                    | 0.58 ± 0.12                 | 191                         | 5.9 ± 0.2                      | 10                          |

\(a\) For hTDG (or hTDG\textsuperscript{cat}) alone.

\(b\) Steady-state turnover of hTDG (or hTDG\textsuperscript{cat}) in the presence of hAPE1.

of hAPE1 (500 nM). We found that the maximal rate of AP product formation is essentially unchanged in the presence of hAPE1, \(k_{\text{max}} = 2.0 ± 0.2 \text{ min}^{-1}\) (data not shown). Similarly, hAPE1 (500 nM) did not increase \(k_{\text{max}}\) for hTDG\textsuperscript{cat} acting upon the G-U20 substrate (not shown). Thus, hAPE1 did not influence steps of the hTDG reaction before product release, indicating the stimulation of hTDG turnover by hAPE1 involves product release (and/or a conformational change in hTDG and/or AP-DNA required for product release). We note that the 500 nM hAPE1 concentration used for these single turnover experiments is high, ~10-fold higher than that needed to realize the maximal hAPE1 effect on hTDG turnover, as shown below.

We also repeated the burst kinetics experiments for hTDG (200 nM) and the G-U20 substrate (2000 nM), this time in the presence of hAPE1, with concentrations ranging from 0 to 50 nM (Fig. 6). We found that hAPE1 does not significantly alter the rate or amplitude of the exponential burst phase, consistent with the conclusion from single turnover experiments that hAPE1 did not affect steps of the hTDG reaction before product release. However, a significant increase was observed for the steady-state phase, indicating that hAPE1 increases the turnover of hTDG by increasing the rate of product release.

hAPE1 Dramatically Increases hTDG Turnover—To rigorously quantify the effect of hAPE1 on the steady-state turnover of hTDG (i.e. its effect on \(k_{\text{cat}}\)), we developed a coupled enzyme (hTDG-hAPE1) fluorescence assay (Fig. 2). The DNA substrates were designed with the molecular beacon approach (38, 39), where the sequential activity of hTDG and hAPE1 generates a large fluorescence increase, and the coupled reaction is monitored in real-time. As expected for a properly executed coupled-enzyme assay (36), the rate constant obtained from the hTDG-hAPE1 assay reflects the steady-state turnover of the primary enzyme, hTDG, and is independent of the coupling enzyme, hAPE1, because we use a sufficiently high concentration of hAPE1 to ensure that hTDG turnover is rate-limiting. Importantly, the steady-state activity of hAPE1 alone, \(k_{\text{cat}} > 60 \text{ min}^{-1}\) (37), is at least 10,000-fold greater than that reported here for hTDG alone (Table 1). Nevertheless, because the rate-limiting step of the coupled-enzyme reaction is likely to be hAPE1-stimulated dissociation of the hTDG product complex, it was necessary to determine the amount of hAPE1 required to obtain the maximal stimulatory effect such that the observed rate constants are independent of hAPE1 concentration (Fig. 2D).

The utility of our coupled-enzyme assay for determining the steady-state kinetic parameters of the hTDG reaction, as stimulated by hAPE1, is illustrated by our results for hTDG\textsuperscript{cat} acting upon the G-U20 substrate (Fig. 7). Using the coupled-enzyme assay, we determined the steady-state rate constant as a function of G-U20 substrate concentration (Fig. 7) and fitted these data to the Michaelis-Menten equation (Equation 3), yielding...
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$k_{\text{cat}}^{\text{APE1}} = 0.46 \pm 0.02 \text{ min}^{-1}$ and $K_m = 162 \pm 17 \text{ nM}$ (where $k_{\text{cat}}^{\text{APE1}}$ denotes $k_{\text{cat}}$ for hTDG in the presence of hAPE1). A comparison of the rate constants for the steady-state turnover of hTDG$^{\text{cat}}$ alone ($k_{\text{cat}}$) and in the presence of hAPE1 ($k_{\text{cat}}^{\text{APE1}}$) reveals that hAPE1 enhances the turnover of hTDG$^{\text{cat}}$ for G-U20 by 77-fold (Table 1). In fact, the hAPE1 effect on product release is so large that $k_{\text{cat}}^{\text{APE1}}$ approximates the maximal rate of AP-DNA product formation, $k_{\text{max}} = 0.9 \pm 0.1 \text{ min}^{-1}$. Thus, in the presence of hAPE1, product release is no longer fully rate-limiting for hTDG$^{\text{cat}}$ acting upon the G-U substrate.

We also used the coupled-enzyme assay to examine the effect of hAPE1 on the steady-state turnover of full-length hTDG for the G-U20 substrate. The hAPE1-stimulated turnover of hTDG is lower than that of hTDG$^{\text{cat}}$ for G-U20, which was somewhat unexpected given that $k_{\text{max}}$ is 2-fold greater for hTDG versus hTDG$^{\text{cat}}$ (Table 1). This precluded accurate measurements at low concentrations of G-U20 substrate (i.e. below 50 nM). Nevertheless, the data provide an accurate value for the maximal hAPE1-stimulated turnover of hTDG, $k_{\text{cat}}^{\text{APE1}} = 0.13 \pm 0.01 \text{ min}^{-1}$, and an estimated Michaelis constant of $K_m = \approx 10 \text{ nM}$ (not shown). A comparison of $k_{\text{cat}}$ and $k_{\text{cat}}^{\text{APE1}}$ reveals that hAPE1 enhances hTDG turnover by a remarkable 26-fold for the G-U substrate. Although this hAPE1 effect is large, the observation that $k_{\text{max}}^{\text{APE1}}$ exceeds $k_{\text{cat}}$ by 14-fold indicates product release is still rate-limiting for hTDG processing of G-U substrates, even in the presence of hAPE1.

We also examined the hAPE1-stimulated turnover of hTDG for the G-T substrate using the coupled-enzyme assay. The coupled reaction was slow, precluding rate measurements at low substrate concentrations. Nevertheless, rate constants were determined for substrate concentrations of 750, 1000, and 1500 nM and were found to be essentially the same (not shown), indicating saturating substrate conditions. Accordingly, the average of these data is taken as the maximal steady-state rate constant, $k_{\text{cat}}^{\text{APE1}} = 0.014 \pm 0.001 \text{ min}^{-1}$ (Table 1). Thus, hAPE1 increases hTDG turnover by 42-fold for the G-T substrate. The observation that $k_{\text{cat}}^{\text{APE1}}$ is merely 6-fold lower than $k_{\text{max}}$ indicates product release is much less rate-limiting in the presence of hAPE1 for hTDG processing of the G-T substrate. Notably, we find that a 100 nM concentration of hAPE1 provides the maximal stimulatory effect on hTDG turnover for the G-T substrate (not shown).

We note that the stimulatory effects of hAPE1 observed here are much larger than those reported previously for other DNA glycosylases. hAPE1 increases the turnover of hUNG by 2–4-fold (6, 46) and has a 4-fold effect on the turnover of human 8-oxoguanine DNA glycosylase (hOGG1) (9).

Kinetics for a G-FU Substrate—Given the much higher $k_{\text{max}}$ observed previously for G-FU relative to G-U substrates (14), it was of interest to examine the enzymatic turnover ($k_{\text{cat}}$) of hTDG for a G-FU substrate and the potential stimulatory effect of hAPE1. For hTDG alone and G-FU20, the maximal rate of product formation, $k_{\text{max}} = 278 \pm 35 \text{ min}^{-1}$, was 440-fold greater than steady-state turnover, $k_{\text{cat}} = 0.63 \pm 0.17 \text{ min}^{-1}$ (Fig. 8A, Table 1), indicating $k_{\text{cat}}$ is limited by product release. Using our coupled-enzyme assay, we obtained $k_{\text{cat}}^{\text{APE1}} = 0.54 \pm 0.03 \text{ min}^{-1}$ and $K_m = 39 \pm 8 \text{ nM}$ (Fig. 8B). Thus, hAPE1 does not enhance the turnover of hTDG for the G-FU substrate.

Given that $k_{\text{cat}}$ is already quite fast for G-FU20 (~100-fold faster than G-U20), this may reflect an upper limit for the effect of hAPE1 on the dissociation of AP-DNA from hTDG (i.e. $k_{\text{cat}}^{\text{APE1}} \approx 0.54 \text{ min}^{-1}$).

For hTDG$^{\text{cat}}$ and the G-FU20 substrate, we find $k_{\text{max}} = 111 \pm 14 \text{ min}^{-1}$ (not shown) and $k_{\text{cat}} = 0.58 \pm 0.12 \text{ min}^{-1}$ (Fig. 8C). The observation that $k_{\text{max}}$ exceeds $k_{\text{cat}}$ by 191-fold again indicates rate-limiting product release. Using the coupled enzyme assay we find $k_{\text{cat}}^{\text{APE1}} = 5.9 \pm 0.2 \text{ min}^{-1}$ and $K_m = 59 \pm 7 \text{ nM}$ (Fig. 8D). Thus, hAPE1 increases the turnover of hTDG$^{\text{cat}}$ by 10-fold for the G-FU substrate, a smaller effect than observed for the G-U substrate (77-fold). This may be explained in part by the fact that turnover of hTDG$^{\text{cat}}$ alone is ~100-fold faster for G-FU relative to G-U (Table 1). The faster turnover observed for G-FU relative to G-U substrates has implications for the mechanism of hTDG, as discussed below.

DISCUSSION

Implications for the Kinetic Mechanism of hTDG—The pre-steady-state kinetics experiments collected here for hTDG (and hTDG$^{\text{cat}}$) provide important new insight into its catalytic mechanism. hTDG is widely regarded as a “single turnover” enzyme, because in many previous studies the reaction reaches a plateau as the [product]/[enzyme] ratio approaches unity for G-U and G-T substrates (13, 27). Nevertheless, steady-state turnover ($k_{\text{cat}}$) can be measured using pre-steady-state multiple turnover (burst kinetics) experiments, collected with a high enzyme concentration and excess substrate (Fig. 5, A and C). We find hTDG turnover is very slow for the G-U substrate, $k_{\text{cat}} = 0.005 \pm 0.001 \text{ min}^{-1}$, and strikingly slow for the G-T substrate, $k_{\text{cat}} = 0.00034 \pm 0.00007 \text{ min}^{-1}$, corresponding to a half-life of 34 h for dissociation of the product complex.
Our results provide the first comparison of single turnover ($k_{\text{max}}$) and steady-state ($k_{\text{cat}}$) activity of hTDG reported for any substrate. In all cases (G-T, G-U, and G-FU substrates), we find $k_{\text{max}} \gg k_{\text{cat}}$ with $k_{\text{max}}/k_{\text{cat}}$ ranging from 150 to 440 (Table 1). The very large difference in these rate constants confirms the rate-limiting step(s) of the hTDG reaction occurs after the chemical step (35), i.e. product release, which includes dissociation of AP-DNA and the excised base and may require a conformational change for hTDG and/or AP-DNA (Fig. 4).

Previous studies and our unpublished observations indicate that release of AP-DNA, rather than release of the excised base, is the rate-limiting step of product release. AP-DNA binds tightly to hTDG (29), dissociates very slowly from the binary hTDG-AP-DNA complex (7), and is a potent inhibitor of hTDG (7, 27, 29). In contrast, we and others find hTDG is not inhibited by the nucleobases that it removes from DNA (including uracil, thymine, FU, and cC), even at concentrations of up to 5 mM (17), indicating they do not bind hTDG with significant affinity. Although these results indicate AP-DNA dissociation is rate-limiting, they do not indicate the order of product release, i.e. whether the base dissociates before or after AP-DNA (Fig. 4). For MutY, which excises adenine mismatched with 8-oxoguanine, adenine dissociates rapidly, $k_{\text{off}} > 5 \text{ min}^{-1}$, much faster than AP-DNA, $k_{\text{off,AP}} \approx 0.005 \text{ min}^{-1}$ (40, 41, 47). For mismatch-specific uracil glycosylase, the *E. coli* ortholog of hTDG that also exhibits rate-limiting product release (48), an “escape route” for release of uracil before AP-DNA was suggested by a crystal structure (49), although this has not been confirmed. Our findings suggest a different mechanism for hTDG.

The burst kinetics experiments collected here indicate the excised base influences the dissociation rate of AP-DNA from the hTDG product complex. Our finding that $k_{\text{cat}}$ for G-FU20 is 126- and 1850-fold faster than $k_{\text{cat}}$ for G-U20 and G-T20 (Table 1) indicates a similarly large difference in the rate-limiting dissociation of AP-DNA (because $k_{\text{off,AP}} \approx k_{\text{cat}}$). These striking differences were not expected, given that the AP-DNA product is identical, and they suggest the excised base remains trapped in the product complex by AP-DNA. Consistent with this idea, our recent crystal structure of hTDGcat bound to abasic DNA reveals no obvious pathway for departure of the excised base prior to AP-DNA absent a significant enzyme conformational change (16).

One explanation for the large differences in $k_{\text{off,AP}}$ for the G-T, G-U, and G-FU reactions is that the ionization state of the excised base may differ when bound in the product complex. Our previous studies indicate the hTDG reaction is highly dissociative and that the departing base is negatively charged, indicating the active site stabilizes the anionic base to some extent (14). The pK$_{a}$ (N1 nitrogen) is much lower for FU (pK$_{a}$N1 = 8.43) relative to U (pK$_{a}$N1 = 9.76) and T (pK$_{a}$N1 = 10.19); thus, FU is more likely than U (and U more likely than T) to be anionic in the product complex. The anionic base may promote the dissociation of AP-DNA by repulsive interactions with the DNA phosphates. This idea could be examined by determining the ionization state of FU, U, and T in the ternary product complex using NMR spectroscopy (50). Such experiments unexpectedly revealed that uracil is anionic when bound in the product complex of uracil DNA glycosylase at neutral pH (50).

**Mechanism for hAPE1 Stimulation of hTDG**—Previous studies of BER for organisms ranging from *E. coli* to humans have shown that AP endonucleases enhance the turnover of DNA glycosylases (6–10, 51, 52). A consistent observation is that the AP endonuclease affects product release rather than the chemical step of the glycosylase reaction. Although the detailed molecular mechanism has remained unknown, two basic ideas have emerged. For the passive enhancement mechanism, the AP endonuclease stimulates glycosylase turnover by simply depleting the concentration of AP-DNA, converting it to 5’-nicked AP-DNA, which should be less inhibitory to the glycosylase (8). The active displacement mechanism requires that the AP endonuclease interacts with the glycosylase and/or AP-DNA to disrupt the product complex (6) and may also involve endonucleolytic depletion of AP-DNA. A passive mechanism was proposed for hAPE1 stimulation of hOGG1 (human 8-oxoguanine DNA glycosylase) (9, 51), although a recent study suggests active displacement may contribute (42). An active mechanism was proposed for hAPE1 stimulation of hUNG (6), and hAPE1 stimulation of human MutY homolog appears to involve protein-protein interactions (53, 54). Active displacement is indicated for AP endonuclease (Exo III and Endo IV) stimulation of MutY in *E. coli* (10). For the hAPE1 stimulation of hTDG, both passive (13, 31) and active (6, 7, 17, 30, 32) mechanisms have been proposed.

Our findings demonstrate that hAPE1 stimulates hTDG turnover by active displacement of AP-DNA, as illustrated by our results for the G-U substrate. The burst kinetics experiments provide a good estimate for the spontaneous AP-DNA dissociation rate for the G-U reaction, where $k_{\text{off,AP}} \approx k_{\text{cat}} = 0.005 \text{ min}^{-1}$ (true because $k_{\text{max}} \gg k_{\text{cat}}$). Meanwhile, the coupled-enzyme assay provides a lower limit for the hAPE1-stimulated dissociation rate of AP-DNA from the hTDG product complex ($k_{\text{off,AP}} \geq k_{\text{cat}} = 0.13 \text{ min}^{-1}$). This is true because, by definition, no individual step of the coupled-enzyme reaction can be slower than the observed steady-state rate constant ($k_{\text{cat}}(\text{APE1})$). Our results exclude the passive displacement mechanism, because the spontaneous AP-DNA dissociation rate ($k_{\text{off,AP}} \approx 0.005 \text{ min}^{-1}$) is not kinetically competent with the hAPE1-stimulated turnover rate ($k_{\text{cat}}(\text{APE1}) = 0.13 \text{ min}^{-1}$). The same argument applies to hTDG acting upon the G-T substrate ($k_{\text{off,AP}} \approx 0.00034 \text{ min}^{-1}$ and $k_{\text{cat}}(\text{APE1}) = 0.014 \text{ min}^{-1}$) and hTDGcat processing the G-U substrate ($k_{\text{off,AP}} \approx 0.006 \text{ min}^{-1}$ and $k_{\text{cat}}(\text{APE1}) = 0.46 \text{ min}^{-1}$). Active displacement is required, because hAPE1 increases the dissociation rate of AP-DNA, presumably by contacting hTDG and/or AP-DNA to disrupt the product complex.

Other evidence supports an active displacement mechanism. The observation that $k_{\text{cat}}$ (hence $k_{\text{off,AP}}$) is identical for hTDGcat and hTDG (Table 1), whereas the hAPE1-effect is greater for hTDGcat (Table 1) indicates active displacement, because one would expect the same hAPE1 effect for a passive displacement mechanism (i.e. if hAPE1 does not alter $k_{\text{off,AP}}$). Active displacement is also consistent with the previous observation that Endo IV, an *E. coli* AP endonuclease that is structurally unrelated to hAPE1, does not stimulate hTDG for G-T or G-cC substrates (7, 30). If a passive mechanism prevailed, i.e. depletion of sponta-
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neously released AP-DNA, one would expect Endo IV (or any AP endonuclease) to stimulate hTDG turnover.

hAPE1 Effect Depends on AP-DNA Release Rate—Previous studies and our findings suggest that when the spontaneous dissociation rate of AP-DNA ($k_{\text{off,AP}}$) is increased (i.e. due to enzyme or substrate modifications), a lower concentration of hAPE1 provides the maximal stimulatory effect. For example, AP-DNA dissociates more rapidly from SUMO-modified hTDG than from hTDG, and a 5 nM concentration of hAPE1 increased the turnover of SUMO-hTDG but had no effect on turnover of unmodified hTDG (for a G-U substrate) (32). Similarly, $k_{\text{off,AP}}$ is faster for C-AP-DNA versus G-AP-DNA, and the stimulatory effect of 10 nM hAPE1 on hTDG turnover is much greater for C-U versus G-U substrates (7). We find that $k_{\text{off,AP}}$ is much faster for G-FU relative to G-U substrates (indicating faster $k_{\text{off,AP}}$ for the G-FU reaction), and the concentration of hAPE1 required for maximal enhancement is lower for G-FU (25 nM) versus G-U (50 nM). Similarly, a 100 nM concentration of hAPE1 provides maximal enhancement of hTDG turnover for the G-T substrate, and $k_{\text{off,AP}}$ is lower for G-T relative to G-U. These observations also indicate that hAPE1 interacts with the hTDG product complex to disrupt it (i.e. active displacement) and that the interaction is more likely to be productive if the inherent AP-DNA release rate ($k_{\text{off,AP}}$) is faster. They are not consistent with passive enhancement because more rather than less hAPE1 should be required when $k_{\text{off,AP}}$ is faster. Further studies are needed to uncover the mechanistic underpinnings of these intriguing observations.

Nature of the Stimulatory Interaction—Our finding that hAPE1 enhances hTDG$^{\text{cat}}$ turnover by 77-fold (for G-U20) reveals that any stimulatory interactions with hAPE1 do not require the N- or C-terminal domains of hTDG (residues 1–110 or 309–410). Indeed, the smaller hAPE1-effect for hTDG versus hTDG$^{\text{cat}}$ (Table 1) indicates the N- and/or C-terminal regions tend to diminish the stimulatory effect of hAPE1.

Previous studies using yeast two-hybrid and electrophoretic mobility shift experiments found no evidence for a stable bimolecular interaction for hTDG and hAPE1 (7, 30). Attempts to visualize a stable complex of hTDG, AP-DNA, and hAPE1 using electrophoretic mobility shift and surface plasmon resonance were also unsuccessful (7, 30). This is perhaps not surprising because hAPE1 displaces hTDG and rapidly converts AP-DNA to 5'-nicked AP-DNA. The stimulatory interactions of hAPE1 with hTDG and/or AP-DNA are probably transient and weak and may involve selective recognition of the hTDG product complex rather than free hTDG (7). Indeed, a stable complex seems incompatible with the robust stimulation of hTDG (and hTDG$^{\text{cat}}$) observed here.

Nevertheless, a stable interaction was reported for murine TDG and APE1 (using glutathione S-transferase pulldown assays) involving residues 92–121 of mTDG (55). This region of mTDG is acetylated (Lys residues) by the transcriptional co-activator CBP/p300, and acetylation of mTDG disrupted its bimolecular interaction with mAPE1 (55). The corresponding (nearly identical) acetylation domain of hTDG, residues 81–110, is not present in hTDG$^{\text{cat}}$. Yet, we find that hTDG$^{\text{cat}}$ is strongly stimulated by hAPE1, 77-fold for the G-U substrate. Clearly, the acetylation domain of hTDG is not required for stimulatory interactions with hAPE1, a finding that conflicts with the previous suggestion that acetylation of TDG regulates the recruitment of APE1, hence the second step of BER (55). Our results indicate that any hTDG-hAPE1 interaction mediated by the acetylation domain, if adopted in vivo, may be important for some function other than promoting hTDG turnover. Additional experiments are warranted to determine the effect of acetylation on the turnover of hTDG in the presence and absence of hAPE1.

How Might hAPE1 Actively Stimulate hTDG?—Our finding that hAPE1 actively disrupts the hTDG product complex raises the question of exactly how this occurs. It was suggested that hAPE1, which forms extensive minor groove interactions, stimulates the turnover of hUNG by binding the minor groove and processing toward hUNG to "pry" it from the AP site (6, 56). Such a mechanism seems consistent with our findings, given some limitations. The 19-bp DNA substrates used here (Fig. 1) are not much longer than the overall footprint (~12 bp) for one hTDG$^{\text{cat}}$ subunit, which binds the minor groove and occupies less than half of the total circumference of the helix (16). Thus, hAPE1, with a footprint of about 8 bp (56, 57), could bind the minor groove immediately adjacent to hTDG and on 3’ side of the AP site (Fig. 9). This would position hAPE1 to initially contact the "insertion loop" of hTDG, which plays key roles in
substrate recognition and nucleotide flipping and accounts for most of the DNA contacts formed by hTDG (16), and may also disrupt the DNA phosphate contacts involving Lys-246 and Lys-248 (Fig. 9). Disruption of the insertion loop and/or the Lys-246/Lys-248 interactions could conceivably promote dissociation of the product complex. hAPE1 binding to the 5′ side of the AP site may be precluded by the other (nonspecific) hTDG subunit if the product complex involves 2:1 (protein:DNA) binding (Fig. 9) as indicated by our recent structural and biochemical studies (16). Moreover, hAPE1 binding 5′ to the AP site is not consistent with observation that the hAPE1 effect is the same for G-U20 and a shorter G-U17 substrate (not shown). G-U17 is identical to G-U20 (Fig. 1) but has just three base pairs located 5′ to the AP site, offering very little foothold for binding of hAPE1 5′ to the AP site.

Of course, other active displacement mechanisms are possible. A direct handoff of AP-DNA from hTDG to the active site of hAPE1 seems unlikely because both enzymes flip the abasic deoxyribose deep into their active site and contact the same five phosphates flanking the AP site. However, hAPE1 could potentially contact hTDG alone to promote dissociation of the product complex, and then compete with hTDG to bind freely released AP-DNA. Additional biochemical and structural studies are needed to fully elucidate the mechanism by which hAPE1 disrupts the hTDG product complex.

Implications for SUMO Modification of hTDG—hTDG binds to and is covalently modified by SUMO-1 and SUMO-2/3, which decreases its DNA binding affinity, apparently by stabilizing an α-helix (residues 317–329) that clashes with DNA (32, 45). For G-U substrates, k_{cat} is higher for SUMO-hTDG versus hTDG, due likely to faster AP-DNA product release, and the stimulatory effect of hAPE1 is greater for SUMO-hTDG (32).

Consistent with weaker substrate binding, a 5 mM concentration of SUMO-hTDG exhibited no G-T activity, but it was not reported whether G-T activity could be recovered at higher enzyme concentrations. It was suggested that product-bound hTDG is SUMOylated to increase the dissociation rate of AP-DNA and that the SUMO group is subsequently removed (32). Our studies provide a measure of the hAPE1-stimulated turnover of hTDG for a G-T substrate, k_{cat}^{hAPE1} = 0.014 min^{-1}, which corresponds to a half-life of ~50 min for dissociation of the product complex. Assuming a similar rate in vivo, our finding suggests ample time and a potential need for SUMOylation of hTDG to further enhance the effect of hAPE1 on product release for processing G-T mispairs.

SUMOylation of hTDG seems less important for G-U processing, because the hAPE1-stimulated turnover is relatively fast (k_{cat}^{hAPE1} = 0.13 min^{-1}). Additional studies are needed to quantitatively establish the effect of TDG SUMOylation on product release in the presence and absence of hAPE1.

Studying hTDG Using Steady-state Kinetics—In addition to illuminating the mechanism for hAPE1 stimulation of hTDG, our coupled-enzyme fluorescence assay provides a method for monitoring steady-state kinetics of hTDG in real time. This assay could potentially be useful for screening and evaluating hTDG inhibitors, determining the damaging effect of removing specific side chains of hTDG by site-directed mutagenesis and structure-activity correlations using modified substrates. Such studies may be most productive by using hTDG-cat and the G-FU20 substrate, which provide the highest turnover (k_{cat}^{hAPE1} = 6 min^{-1}), hence the largest dynamic range for determining the effect of enzyme or substrate modifications.

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