Role of Two Strictly Conserved Residues in Nucleotide Flipping and N-Glycosylic Bond Cleavage by Human Thymine DNA Glycosylase*

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Thymine DNA glycosylase (TDG) promotes genomic integrity by excising thymine from mutagenic G-T mismatches arising by deamination of 5-methylcytosine, and follow-on base excision repair enzymes restore a G-C pair. TDG cleaves the N-glycosylic bond of dT and some other nucleotides, including 5-substituted 2'-deoxyuridine analogs, once they have been flipped from the helix into its active site. We examined the role of two strictly conserved residues; Asn140, implicated in the chemical step, and Arg275, implicated in nucleotide flipping. The N140A variant binds substrate DNA with the same tight affinity as wild-type TDG, but it has no detectable base excision activity for a G-T substrate, and its excision rate is vastly diminished (by ~104-fold) for G-U, G-FU, and G-BrU substrates. Thus, Asn140 does not contribute substantially to substrate binding but is essential for the chemical step, where it stabilizes the transition state by 6 kcal/mol (compared with 11.6 kcal/mol stabilization provided by TDG overall). Our recent crystal structure revealed that Arg275 penetrates the DNA minor groove, filling the void created by nucleotide flipping. We found that the R275A and R275L substitutions weaken substrate binding and substantially decrease the base excision rate for G-T and G-BrU substrates. Our results indicate that Arg275 promotes and/or stabilizes nucleotide flipping, a role that is most important for target nucleotides that are relatively large (dT and bromodeoxyuridine) and/or have a stable N-glycosylic bond (dT). Arg275 does not contribute substantially to the binding of TDG to abasic DNA product, and it cannot account for the slow product release exhibited by TDG.

The chemically reactive bases in DNA are continuously modified by oxidation, alklylation, and deamination, creating mutagenic and cytotoxic lesions that are implicated in aging and diseases including cancer (1, 2). Such damage is handled predominantly by the base excision repair pathway, initiated by one of many damage-specific DNA glycosylases (3). These enzymes use a nucleotide flipping mechanism to find damaged bases within the vast excess of normal DNA and cleave the base-sugar (N-glycosylic) bond to release the base, and follow-on base excision repair proteins complete the repair process.

Thymine DNA glycosylase (TDG)2 removes thymine from mutagenic G-T mismatches, one of the few glycosylases that removes a normal base from DNA. Consistent with the need to avoid acting upon undamaged DNA, TDG activity is 18,000-fold greater for G-T mismatches relative to A-T pairs (4). TDG is also specific for a particular DNA sequence, exhibiting the highest activity for G-T mismatches (and other lesions) with a 5'-flanking C-G base pair, i.e. damage located in CpG site (4–7). Because cytosine methylation in vertebrates occurs selectively at CpG sites, the specificity of TDG indicates its predominant biological substrate is a G-T mispair arising from deamination of 5-methylcytosine (8).

Cytosine methylation at CpG sites is central for regulating gene expression and maintaining genomic stability (9). Another human DNA glycosylase has specificity for G-T mismatches arising at CpG sites, methyl-binding domain IV (10–12), suggesting a biological imperative to maintain the integrity of CpG sites. Nevertheless, CpG sites exhibit a disproportionately high frequency of mutations (C → T) in human cancers and genetic disease (13–15), and it was suggested that the slow enzymatic turnover of TDG may contribute (16). An alternative explanation is raised by reports that TDG may participate in the demethylation of CpG sites by processing G-T mispairs created by active deamination of 5-methylcytosine (17, 18), which would dramatically increase the burden of G-T mispairs. A preliminary report that homozygous knock-out of the TDG gene in mice results in embryonic lethality is consistent with such a role (19). Although these findings remain to be substantiated, they underscore the importance of understanding the catalytic mechanism of TDG.

TDG is relatively permissive and can remove 5-halogenated uracils (5-fluouracil, 5-chlorouracil, 5-bromouracil, and 5-iodouracil), many other 5-substituted uracils, 3N4-ethenocytosine, hypoxanthine, and other damaged bases (see Ref. 4 and references therein). The CpG sequence specificity is retained

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The abbreviations used are: TDG, human thymine DNA glycosylase; ITC, isothermal titration calorimetry; MUG, mismatch specific uracil glycosylase; \( T' \), \( 2' \)-deoxy-2'-fluorouracil; \( U' \), \( 2' \)-deoxy-2'-fluorothymidine; UDGD, uracil DNA glycosylase; BrdU, bromodeoxyuridine; HPLC, high pressure liquid chromatography; CpG, dC-dG dinucleotide; FdU, 5-fluoro-2'-deoxyuridine; dU, 2'-deoxyuridine; dT, 2'-deoxythymidine; FU, 5-fluorouracil; BrU, 5-bromouracil.
for these substrates, consistent with the conclusion that CpG-T is the predominant biological substrate (8). Nevertheless, comparing the kinetic and binding parameters for a select group of substrates can provide much insight into the catalytic mechanism of TDG (4, 20, 21), and such an approach was used here. We previously observed robust activity for the excision of 5-halogenated uracils, including FU, CIU, and BrU, even from DNA sequence contexts other than CpG, with implications for the catalytic mechanism of TDG and for the cytotoxicity associated with the incorporation of these bases in DNA (4). Consistent with the potent in vitro activity for excising FU (4, 20–22), a recent study finds that FU excision by TDG contributes to the DNA-mediated toxicity of FU in human and mouse cancer cells (23). Detailed studies of the TDG catalytic mechanism should further illuminate its ability to promote the toxicity of FU, a widely used anticancer agent. Toward this end, we have investigated the role of two strictly conserved active site residues of TDG, Asn140 and Arg275.

Together, human TDG and the Escherichia coli mismatch-specific uracil DNA glycosylase (MUG, 32% identical) are the most thoroughly characterized members of the TDG-MUG family (5, 19, 24), part of the uracil DNA glycosylase (UDG) superfamily (1, 25). We investigated the catalytic role of Asn140, which is found in a motif (138GINPG142) that is strictly conserved in TDG-MUG enzymes (25). Previous structural and biochemical studies indicate that Asn140 is important, probably for the chemical step (22, 26–29), yet its catalytic role had not been rigorously investigated for any member of the TDG-MUG family. To address this deficiency, we examined the role of Asn140 by determining the effect of the N140A substitution on substrate binding and the base excision rate for a number of different substrates.

All of the DNA glycosylases employ nucleotide flipping to extrude the target nucleotide from the helix and gain access to the damaged base and the scissile N-glycosyl bond (30). Most glycosylases use a bulky side chain (Asn, Arg, Gln, Leu, or Tyr) to plug the helical space created by nucleotide flipping, although exceptions include 3-methyladenine DNA glycosylase, which has a Gly at this position (31). These “nucleotide flipping” residues can promote forward progress of the target nucleotide toward the active site and/or hinder its reverse flipping back into the helix, increasing its lifetime in the active site (30, 32, 33). A structure of TDG bound to abasic DNA shows that the Arg275 side chain penetrates the minor groove, plugging the space vacated by the flipped base and contacting two DNA backbone phosphates that flank the target site (Fig. 1) (29). Although Arg275 is strictly conserved in TDG from vertebrates and lower eukaryotes (19), the closely related MUG enzymes have a Leu in the corresponding position, as does the more distantly related UDG enzymes. We have examined the catalytic role of Arg275 by determining the effect of R275A and R275L substitutions on substrate binding, the base excision rate ($k_{max}$), and the binding and release of abasic DNA product.

**EXPERIMENTAL PROCEDURES**

**Materials**—The DNA used for this work is shown in Fig. 2. Duplex DNA was hybridized by rapid heating to 80 °C followed by slow cooling to room temperature. DNA oligonucleotides were synthesized at the Keck Foundation Biotechnology Resource Laboratory of Yale University (trityl-on), purified using Glen-Pak purification cartridges (Glen Research) following the manufacturer’s instructions, and quantified by absorbance (260 nm) as previously described (20). Purity was verified by analytical anion exchange HPLC under denaturing (pH 12) conditions (21). Phosphoramidites for the noncanonical DNA nucleotides were obtained from Glen Research. The phosphoramidite used for incorporation of 2′-deoxy-2′-fluoroarabinouridine (U$_F$; Fig. 2) was obtained from ChemGenes Corp. (Wilmington, MA), and the phosphoramidite for 2′-deoxy-2′-fluoroarabinohymidine (T$_F$) (34) was obtained from Link Technologies (Lanarkshire, UK). These phosphoramidites were incorporated using standard phosphoramidite chemistry with coupling times extended to 5 min. Control experiments demonstrate that duplex DNA containing U$_F$ or T$_F$ (paired with guanine) are not cleaved by TDG when incubated for an
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extended time (>48 h) under single turnover conditions, with a DNA concentration of 0.5 \textmu M and an enzyme concentration of 5 \textmu M (not shown), consistent with previous findings for TDG (35) and MUG (27). Previous studies show that a single T\textsuperscript{F} substitution slightly elevates the DNA duplex melting temperature (T\textsubscript{m} \text{in} \text{creased} \sim 0.6 ^\circ C) and does not significantly alter the overall structure of B-DNA. For the T\textsuperscript{2}-fluorouracil substitution stabilizes an O4'-endo (east) sugar pucker that is compatible with B-DNA geometry (36).

Human thymine DNA glycosylase (TDG) was expressed and purified as previously described (4, 29), quantified by absorbance (\text{\epsilon}_{280} = 31.5 \text{ M}^{-1} \text{ cm}^{-1}), flash frozen, and stored at \sim 80 ^\circ C. Expression plasmids for the N140A, R275A, R275L, and N140A/R275L variants were generated by PCR from the pET-28 expression plasmid for wild-type enzyme using the QuikChange mutagenesis protocol (Stratagene) with the following PCR primers: N140A forward, 5' -GTC ATT ATT GGC CAT TAG TCC CGG GCC GGA CTA ATG GCT GC-3'; N140A reverse, 5'-GG AGC CAT TAG TCG CGG TAC GAT GAC-3'; R275A forward, 5'-ATG CCA TCA TCC AGT GCA GCA TGT CCT CGT TTT CCT CG-3'; R275A reverse, 5'-CG AGG AAA CTG AGC ACA TGC TGC ATT ATT GGC (east) sugar pucker that is consistent with B-DNA geometry (36).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Minimal kinetic mechanism for TDG. The association of enzyme and DNA gives a collision complex (E\textcdot S), and nucleotide flipping (involving conformational changes in E and S) gives the reactive enzyme-substrate complex (E\textcdot S'). The chemical step (k\textsubscript{chem}) involves cleavage of the N-glycosidic bond and addition of the nucleophile (water), giving the product complex (E\textcdot B'P). Product release involves a conformational change in E and P (not shown) and is slow. Also indicated are the reaction steps that contribute to the kinetic parameters k\textsubscript{max}, the maximal rate of product formation, and k\textsubscript{cat} (multiple turnover) for substrate (analog).}
\end{figure}

Pre-steady-state Kinetics—Transient kinetics experiments were conducted at 22 \degree C in HEMN.1 buffer (20 \text{mM} HEPES, pH 7.5, 0.1 \text{mM} NaCl, 0.2 \text{mM} EDTA, 2.5 \text{mM} MgCl\textsubscript{2}) with 0.1 mg/ml bovine serum albumin, quenched with 50% (v:v) 0.3 M NaOH, 0.03 \text{mM} EDTA and heated for 15 min at 85 \degree C to induce cleavage of the DNA backbone at abasic sites. The extent of product formation was analyzed using an HPLC assay as described (4, 20). The maximal rate constant for product formation (k\textsubscript{max}) was determined using single turnover experiments collected under saturating enzyme conditions ([E] >> [S] >> K\textsubscript{d}), providing a rate constant that is not impacted by product release or the association of enzyme and substrate (4, 20). The data were fitted by nonlinear regression to Equation 1 using Grafit 5 (37),

\begin{equation}
[\text{Product}] = A(1 - \exp(-k_{\text{obs}}t)) \tag{Eq. 1}
\end{equation}

where A is the amplitude, k\textsubscript{obs} is the rate constant, and t is the reaction time (min). We used a DNA substrate concentration of 0.5 \textmu M and a high enzyme concentration (typically 5 \textmu M) that is 100-fold higher than the K\textsubscript{d} values observed here and reported previously (8, 35). Previous studies in our laboratory (4, 20) indicate that these conditions are saturating and therefore provide the maximal rate constant for product formation (i.e. k\textsubscript{obs} \approx k\textsubscript{max}). This was confirmed by collecting the data at an enzyme concentration of 10 \textmu M, which provided the same rate constant (within experimental error).

The maximal rate constant for enzymatic turnover (k\textsubscript{cat}) was determined using pre-steady-state multiple-turnover kinetics, conducted with a high enzyme concentration (500 nM) and excess substrate (\sim 1 \mu M), where [S] >> [E] >> K\textsubscript{d}, such that k\textsubscript{cat} is not limited by the association of enzyme and substrate (21, 38). Progress curves exhibited “burst” kinetics, with a rapid exponential phase followed by a slow linear phase, indicating that the rate of product formation greatly exceeds that of product release. The data were fitted to Equation 2,

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

where A and k\textsubscript{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\textsubscript{cat}) was obtained by dividing the steady-state velocity (v) by the amplitude (A). In all cases, k\textsubscript{max}/k\textsubscript{cat} > 60, indicating that the rate-limiting step occurs after the chemical step.

Isothermal Titration Calorimetry—ITC experiments were performed using a MicroCal VP-ITC instrument essentially as described previously (29). Protein and DNA samples were dialyzed versus ITC buffer (10 \text{mM} Tris-HCl, pH 7.5, 0.1 \text{mM} NaCl, 0.5 \text{mM} tris-(2-carboxyethyl)phosphine hydrochloride) and degassed prior to data collection. The experiments were collected (at 5 \degree C) by titrating small volumes of DNA (concentration of 15–20 \mu M) into a 1.4-ml sample cell containing enzyme (concentration of 2–3 \mu M). The data were analyzed using pytITC, a python-based program with improved base-line estimates in raw heat data and automatic evaluation of dilution heat as a fitting parameter (29, 39).

RESULTS

Our aim in this work was to probe the catalytic role of two strictly conserved residues of TDG, Asn\textsuperscript{140} and Arg\textsuperscript{275}, using site-directed mutagenesis, pre-steady-state kinetics, and equilibrium binding experiments. As we and others have shown previously, the steady-state turnover of TDG is severely limited by a step following chemistry, i.e. release of the AP-DNA product (7, 21, 40). Accordingly, we used single turnover kinetics experiments under saturating enzyme conditions to determine the effect of Asn\textsuperscript{140} and Arg\textsuperscript{275} substitutions on k\textsubscript{cat}, which reflects the maximal rate of (enzyme-bound) product formation and is not impacted by product release (Fig. 3 (20)).

\textit{Essential Role for Asn\textsuperscript{140}—}We used single turnover kinetics and substrates including G\textsuperscript{T}, G\textsuperscript{U}, G\textsuperscript{FU}, and G\textsuperscript{Br}U to exam-
ine the role of Asn140 in the catalytic mechanism of TDG. We find no evidence of thymine excision from a G-T substrate (0.5 μM) incubated with a saturating concentration of TDG-N140A (10 μM), even after 48 h. The absence of measurable activity is not due to improper folding or instability of the N140A variant, as indicated by results from equilibrium binding studies presented below. Given the high sensitivity of our HPLC assay (20), we can place an upper limit on the activity of TDG-N140A for a G-T substrate, $k_{\text{cat}} < 7 \times 10^{-6}$ min$^{-1}$ (i.e. <1% product in 24 h). Thus, the N140A substitution diminishes the base excision rate by at least $10^{4.4}$-fold (Table 1). We also examined the single turnover activity of TDG-N140A for other substrates. In contrast to a previous report (22), TDG-N140A retains some activity for a G-U substrate, albeit exceedingly low, $k_{\text{cat}} = 0.00011 \pm 0.00001$ min$^{-1}$ (Fig. 4A), revealing a $10^{4.4}$-fold loss in $k_{\text{cat}}$ for the N140A substitution (Table 1). The N140A substitution causes decreases in $k_{\text{cat}}$ of $10^{4.4}$, and $10^{4.1}$-fold for the G-FU and G-BrU substrates, respectively (Fig. 4 and Table 1). These huge effects on $k_{\text{cat}}$ together with the results of binding studies below, indicate an essential role for Asn140 in the chemical step of the TDG reaction.

Asn140 Is Dispensable for Substrate Binding—Given the essential role of Asn140 in base excision, we next sought to determine its role in substrate binding, i.e. in forming the reactive Michaelis complex ($E'$-$S'$ in Fig. 3). To determine the binding affinity ($K_d$) of wild-type TDG to substrate in the absence of base excision, we used DNA containing a 2′-deoxyuridine (dU) analog, U$^f$, which flips into the active site but is resistant to N-glycosyl bond cleavage, as demonstrated previously for human TDG and for MUG and UDG from E. coli (27, 35, 41). As shown in Fig. 5, ITC experiments show that TDG binds the G-U$^f$ substrate analog with high affinity, $K_d = 5 \pm 1$ nM (Table 2). The binding is endothermic, driven by favorable entropy ($\Delta S > 0$) that compensates for large unfavorable enthalpy ($\Delta H > 0$), consistent with our previous results for TDG binding to abasic DNA product (29) and with the thermodynamic signature of other proteins that bind the minor groove of DNA (42). Remarkably, TDG-N140A binds the G-U$^f$ substrate analog with nearly the same affinity and thermodynamic parameters as wild-type TDG (Fig. 5 and Table 2). The exceedingly low base excision activity of TDG-N140A allows a determination of its binding affinity for natural substrate in the absence of base excision (i.e. <1% product formation in 2 h at 5 °C). The affinity of TDG-N140A for a natural G-U substrate, $K_d = 9 \pm 2$ nM, is very similar to that observed for TDG-N140A and wild-type TDG binding to the G-U$^f$ substrate analog (Fig. 5 and Table 2). Our results are consistent with previous findings that U$^f$ is a good mimic of the natural dU substrate (27, 35, 41).

We also examined the binding of TDG and the N140A variant to DNA containing a dT substrate analog, T$^f$. TDG binds the G-T$^f$ substrate analog with high affinity, $K_d = 36 \pm 7$ nM.
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Table 2

| DNA | Enzyme | K\textsubscript{d} (nM) | Fold increase in K\textsubscript{d} | ΔG (kcal/mol) | ΔH (kcal/mol) | TΔS (kcal/mol) |
|-----|--------|----------------|-------------------------------|---------------|---------------|---------------|
| G-U\textsuperscript{T} | TDG | 5 ± 1 | 1.4 | -10.5 ± 0.1 | 36 ± 1 | 46.7 ± 0.9 |
| | N140A | 7 ± 4 | | -10.3 ± 0.2 | 37 ± 1 | 48 ± 1 |
| | R275A | 14 ± 4 | 2.9 | -9.9 ± 0.1 | 34 ± 2 | 46 ± 2 |
| | R275L | 31 ± 3 | 6.3 | -9.55 ± 0.06 | 36 ± 1 | 46 ± 1 |
| G-U | N140A | 9 ± 2 | | -10.2 ± 0.1 | 37.1 ± 0.8 | 47.5 ± 0.6 |
| | N140A/R275L | 50 ± 3 | 5.6 | -9.29 ± 0.03 | 35.2 ± 0.2 | 41.8 ± 0.2 |
| G-T\textsuperscript{F} | TDG | 36 ± 7 | | -9.46 ± 0.1 | 24.7 ± 0.5 | 34.2 ± 0.4 |
| | N140A | 39 ± 2 | 1.1 | -9.43 ± 0.05 | 32.4 ± 0.2 | 41.9 ± 0.2 |
| | R275A | 120 ± 8 | 3.3 | -8.80 ± 0.04 | 14.1 ± 0.1 | 22.9 ± 0.1 |
| | R275L | 130 ± 20 | 3.6 | -8.76 ± 0.08 | 16.7 ± 0.4 | 25.5 ± 0.3 |
| G-T | N140A | 22 ± 1 | | -9.74 ± 0.03 | 30.3 ± 0.3 | 40.1 ± 0.3 |
| | N140A/R275L | 80 ± 20 | 3.6 | -9.0 ± 0.1 | 23.4 ± 0.7 | 32.6 ± 0.6 |
| G-BrU | N140A | 17 ± 2 | 4.4 | -9.87 ± 0.06 | 26.2 ± 0.1 | 36.1 ± 0.1 |
| | N140A/R275L | 74 ± 9 | | -9.08 ± 0.07 | 25.1 ± 0.5 | 34.2 ± 0.1 |
| G-THF | TDG | 9.8 ± 0.3 | | -10.19 ± 0.02 | 29 ± 1 | 39.8 ± 0.9 |
| | R275A | 10 ± 1 | 1 | -10.18 ± 0.01 | 23 ± 2 | 34 ± 2 |
| | R275L | 8.6 ± 0.1 | 0.9 | -10.26 ± 0.01 | 28 ± 1 | 39 ± 2 |

(22).

Our finding that the N140A substitution halts the chemical step while not altering substrate binding indicates the N140A variant is a useful platform for investigating the role of other residues in substrate binding, as illustrated for Arg\textsuperscript{275} below. Although the U\textsuperscript{F} analog has been employed in the study of many DNA glycosylases (27, 35, 41), use of the T\textsuperscript{F} analog has not previously been reported for these enzymes. Our finding that the N140A variant binds with similar affinity and thermodynamic parameters to G-T and G-T\textsuperscript{F} (Table 2) indicates that T\textsuperscript{F} is a good mimic of dT and should be useful in future structural and mechanistic studies of TDG and other glycosylases that excise thymine from DNA.

Effect of Arg\textsuperscript{275} Substitutions on Base Excision—We used single turnover kinetics experiments to determine the effect of Arg\textsuperscript{275} substitutions on k\textsubscript{max} for R275A the steric bulk and positive charge (or at least the polarity) of the Arg side chain are both removed (Fig. 1). Some bulk is retained for R275L, but the shapes of the Leu and Arg side chains differ substantially, and Leu is nonpolar. The R275L variant was also chosen because the related MUG and UDG enzymes have strictly conserved Leu residues in the positions corresponding to Arg\textsuperscript{275} of TDG (25, 26, 29). We used several different target nucleotides to probe the contribution of Arg\textsuperscript{275} to nucleotide flipping and the chemical step of the TDG reaction, which can both impact k\textsubscript{max} (Fig. 3) (4, 20). For the G-FU substrate, the R275A and R275L substitutions give very small effects of 1.6- and 1.4-fold, respectively (Table 1). This indicates essentially no effect on the chemical step for the cleavage of FdU, which is relatively small and is rapidly cleaved by TDG, consistent with our previous findings (4, 20). Likewise, for a G-U substrate, we find small effects on k\textsubscript{max} of 2- and 3-fold for the R275A and R275L substitutions. In contrast, for the G-BrU substrate, k\textsubscript{max} is decreased 4- and 16-fold by the R275A and R275L substitutions, respectively. The largest effects are observed for the G-T substrate, where k\textsubscript{max} is decreased 8-fold for R275A and 30-fold for R275L (Fig. 6 and Table 1). As discussed in detail below, these observations...
indicate that Arg275 serves to promote and/or stabilize nucleotide flipping (i.e. it increases $k_2$ and/or decreases $k_{-2}$; Fig. 3).

**Arg275 Substitutions Decrease Substrate Binding**—We used ITC to examine the effect of R275A and R275L substitutions on binding of TDG to G-U$^F$ and G-T$^F$ substrate analogs (Fig. 7). The R275A and R275L substitutions weaken the binding for G-U$^F$ by 2.9- and 6.3-fold (Table 2). Similarly, the R275A and R275L variants bind 3.3- and 3.6-fold more weakly to the G-T$^F$ substrate analog (Table 2). To examine the effect of the R275L substitution on the binding affinity for natural substrates, we produced the N140A/R275L enzyme, which seems reasonable because the N140A substitution alone halts the chemical step while not significantly altering substrate binding (Tables 1 and 2). The binding affinity for a G-U substrate is 5.6-fold weaker for N140A/R275L versus N140A, the same effect on $K_d$ as observed for binding of R275L to the G-U$^F$ substrate analog (Table 2). Similarly, for the G-T and G-BrU substrates, the binding affinity is about 4-fold weaker for N140A/R275L relative to N140A (Table 2). Because Arg275 is unlikely to contribute to the initial enzyme-substrate (collision) complex (E+S; Fig. 3), the effects of Arg275 substitutions on substrate binding likely reflect a change in nucleotide flipping, i.e. a decrease in $k_2$ or an increase in $k_{-2}$ (Fig. 3), consistent with the effects of Arg275 substitutions on $k_{\text{cat}}$.

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![FIGURE 7. Role of Asn275 in substrate binding. ITC data for G-U$^F$ substrate analog binding to TDG-R275A (A), G-U$^F$ binding to TDG-R275L (B), and natural G-U substrate binding to the N140A/R275L variant (C) are shown. The ITC experiments were collected at 5 °C with enzyme in the sample cell at a concentration of 2–3 μM and DNA in the injection syringe at a concentration of 15–20 μM.](image)

![FIGURE 8. Burst kinetics experiments for TDG and the R275A and R275L variants. Burst kinetics experiments were collected using a 1000 nM concentration of the G-U$^F$ substrate and 500 nM concentration of either TDG (○), TDG-R275A (●), or TDG-R275L (□). The rate of the steady-state phase was not substantially changed by the Arg275 substitutions ($k_{\text{cat}}$ values given in Table 1).](image)

DISCUSSION

**Asn140 Is Essential for the Chemical Step of the TDG Reaction**—Asn140 is strictly conserved in TDG and MUG enzymes, and previous structural and biochemical studies suggested that this
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due to the chemical step of the reaction (26–29), yet its catalytic role had not been rigorously examined for any member of the TDG-MUG enzyme family. Our results demonstrate that Asn\textsuperscript{140} is essential for the chemical step but does not contribute substantially to prior steps, including formation of the reactive enzyme-substrate complex in which the target nucleotide is flipped into the active site (‘E-S’ in Fig. 3).

Indeed, the N140A substitution causes a huge decrease of at least 10\textsuperscript{4}-fold in the base excision rate ($k_{\text{max}}$) for a G-T substrate (Table 1), indicating that Asn\textsuperscript{140} stabilizes the transition state by >6 kcal/mol. For comparison, the total rate enhancement provided by TDG for G-T substrate is $k_{\text{max}}/k_{\text{non}} = 10^{9.5}$, corresponding to 11.6 kcal/mol transition state stabilization (20). A similarly large role for Asn\textsuperscript{140} in the chemical step is observed for the G-U, G-FU, and G-BrU substrates (Table 1). Remarkably, the N140A substitution does not substantially alter the binding affinity of TDG for the G-T\textsuperscript{F} or G-T\textsuperscript{F} substrate analogs (Table 2), indicating that Asn\textsuperscript{140} does not contribute to substrate binding or nucleotide flipping. Similar results were obtained for the corresponding residue in UDG, which is an Asp rather than Asn. The catalytic Asp of UDG stabilizes the transition state by ~5 kcal/mol, compared with 17 kcal/mol transition state stabilization provided by UDG in total (44), and the Asp does not contribute to substrate binding (44). Thus, Asn\textsuperscript{140} of TDG accounts for a larger fraction of transition state stabilization than the catalytic Asp of UDG.

Our findings, together with previous studies, suggest a potential role for this essential Asn of the TDG-MUG enzyme family. Many previous studies show that hydrolysis of the N-glycosyl bond follows a highly dissociative or stepwise mechanism for both enzymatic and noncatalyzed reactions (45). The reactions catalyzed by UDG and MutY (from E. coli) are stepwise, where rupture of the N-glycosyl bond precedes the addition of the nucleophile (water) (46–48). For UDG, N-glycosyl bond cleavage produces a highly unstable glycosyl cation-uracil anion intermediate, and the catalytic Asp appears to play three key roles: stabilizing the glycosyl cation, positioning the nucleophilic water molecule, and activating the nucleophile for attack (via general base catalysis) (44, 49, 50).

A stepwise mechanism seems reasonable for the TDG reaction (20, 45), but the catalytic Asn likely plays a very different role from the Asp of UDG. Our findings and previous studies suggest that the primary role of the essential Asn in TDG-MUG enzymes is to bind and properly position the nucleophilic water molecule. As illustrated in Fig. 9, the crystal structures of TDG and MUG (free enzymes) reveal a water molecule bound to the catalytic Asn via hydrogen bonds to its side chain and backbone carbonyl oxygens, and structures of enzyme-DNA complexes show the Asn would position the water for nucleophilic attack at C1’ of the flipped nucleotide (26, 27, 29). However, unlike the catalytic Asp of UDG, the Asn of TDG cannot activate the nucleophile via general base catalysis, and its side chain carbonyl oxygen (O\text{\textdelta}{1}; Fig. 9) does not appear close enough (to C1’) to stabilize the glycosyl cation (26–29). Thus, for TDG-MUG enzymes the glycosyl cation is likely stabilized by the anionic base (leaving group) and neighboring DNA phosphates, as observed for UDG (48, 51), and potentially by the oxygen of the nucleophilic water molecule.

In the absence of nucleophile activation by a general base, it seems reasonable that a proton may be transferred from the nucleophile to the anionic base, which would also serve to stabilize the leaving group (preventing its reassociation with the glycosyl cation). A recent computational study finds that the noncatalyzed hydrolysis of 2’-deoxyuridine involves transfer of a proton (via intervening water molecules) from the nucleophile to O\text{2} of the uracil anion (52). As noted previously, the structure of the MUG enzyme-substrate complex shows uracil O\text{2} is poised to abstract a proton from the nucleophile (Fig. 9), which could be favorable for the anionic base that results from N-glycosyl bond cleavage (27, 45). Of course, alternative pathways for proton transfer are possible and could potentially involve the backbone carbonyl oxygen of Asn\textsuperscript{140} (Fig. 9). Such a mechanism for nucleophile activation and leaving group stabilization may be important for TDG-MUG enzymes, which do not provide many interactions to stabilize the leaving group or a side chain for general base catalysis (26, 27, 29). The proper positioning of the nucleophile by Asn\textsuperscript{140} may be particularly important for such a mechanism.

Role for Arg\textsuperscript{275} in Nucleotide Flipping—Our results indicate that the Arg\textsuperscript{275} side chain promotes and/or stabilizes nucleotide flipping, and its contribution to base excision is greater for target nucleotides that have a relatively large base (i.e. dT, BrdU) and/or a stable N-glycosyl bond that resists cleavage (such as dT). This is illustrated by considering the differing effects of Arg\textsuperscript{275} substitutions on the base excision rate ($k_{\text{max}}$) for the various substrates used in this work.

We find essentially no effect on $k_{\text{max}}$ for the G-FU substrate, indicating that Arg\textsuperscript{275} does not contribute substantially to transition state stabilization in the chemical step. It also indicates that any effect of the Arg\textsuperscript{275} substitutions on nucleotide flipping ($k_2$ or $k_{\text{-2}}$; Fig. 3) does not diminish the active site lifetime of FdU to an extent that $k_{\text{max}}$ is altered. We note that FdU is the most rapidly cleaved substrate for TDG (Table 1).

In contrast to the absence of an effect for FdU, the R275A and R275L substitutions substantially decrease $k_{\text{max}}$ (by 4- and
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16-fold for the G-BrU substrate (Table 1). To account for this difference, it is important to note that wild-type TDG cleaves FdU 17-fold faster than BrdU (Table 1). As we suggested previously, the difference in wild-type TDG activity is likely due to steric effects (20). BrdU and FdU exhibit the same N-glycosyl bond stability and the same noncatalyzed rate of bond cleavage ($k_{\text{non}}$), but BrdU is much larger and is likely more difficult to flip and/or maintain in the TDG active site than FdU (20). Thus, given the equivalent susceptibility of FdU and BrdU to N-glycosyl bond cleavage (same $k_{\text{non}}$), the much larger effect of Arg\textsuperscript{275} substitutions on $k_{\text{max}}$ for G-BrU versus G-FU substrates indicates an important role for Arg\textsuperscript{275} in promoting and/or stabilizing nucleotide flipping for bulky substrates such as BrdU.

Consistent with this idea, the effects of the Arg\textsuperscript{275} substitutions are greater for G-BrU versus G-U, even though the N-glycosyl bond is much more readily cleaved for BrdU than for dU. Evidently the Arg\textsuperscript{275} substitutions have a substantially greater impact on the active site lifetime of BrdU than dU.

The Arg\textsuperscript{275} substitutions exert the greatest effect on base excision rate ($k_{\text{max}}$) for the G-T substrate (Table 1). Although dT is slightly smaller than BrdU, the N-glycosyl bond of dT is much more resistant to cleavage (20). The significant effect on $k_{\text{max}}$ for both substrates indicates that substitution of Arg\textsuperscript{275} impairs flipping for dT and BrdU nucleotides. The larger $k_{\text{max}}$ effect for dT likely reflects the greater impact of decreased active site lifetime for a nucleotide with a more stable N-glycosyl bond.

The observation that stabilization of nucleotide flipping by Arg\textsuperscript{275} is most important for bulky nucleotides with a stable N-glycosyl bond (dT) is consistent with previous findings that the effects on $k_{\text{max}}$ of altering the CpG context or changing the partner of the target base are largest for cleavage of dT and smallest for FdU (4). Although our findings confirm an important role for Arg\textsuperscript{275} in nucleotide flipping, as predicted by our recent crystal structure, future studies will be required to further elucidate the detailed role of Arg\textsuperscript{275}, i.e. whether it serves to promote forward flipping (increase $k_{\text{f}}$) and/or slow the rate of reverse flipping of the target nucleotide back into the DNA helix (decrease $k_{\text{r}}$).

Leu Is a Poor Substitution for Arg\textsuperscript{275}.—As discussed above, the base excision activity ($k_{\text{max}}$) of TDG for G-T and G-BrU substrates is substantially decreased by the Arg\textsuperscript{275} substitutions. Surprisingly, the activity is ~4-fold lower for the Leu variant (R275L) as compared with the Ala variant (R275A) (Table 1). This result was unexpected because most DNA glycosylases employ a bulky residue to stabilize nucleotide flipping (30) and because the related MUG and UDG enzymes use Leu rather than Arg for nucleotide flipping (25, 26, 29). One potential explanation for the lower activity of R275L versus R275A involves steric hindrance between methyl groups of the Leu side chain and the nucleotides that flank the target nucleotide (Fig. 1B). To explore this idea, we replaced Arg\textsuperscript{275} with Leu in the TDG-AP-DNA crystal structure and produced a model structure for each of the four common Leu rotamers using the program Coot (53). These model structures reveal steric hindrance between the 3’ and 5’-flanking nucleotides and the Leu side chain; the location and extent of the steric clashes vary with the Leu rotamer (not shown). In all models the Leu side chain clashes to some extent with the 3’-dG nucleotide. Importantly, TDG interacts with the 3’-dG to obtain specificity for G-T mismatches and other lesions in a CpG sequence context (5’-CpG/5’-TpG) (29), and perturbing these interactions significantly diminishes base excision activity (4). Thus, replacement of Arg\textsuperscript{275} with Leu could potentially perturb TDG interactions with the 3’-dG. In contrast, the Arg side chain threads nicely through the gap between the 3’- and 5’-flanking nucleotides, and, unlike Leu, the Arg side chain interacts favorably with the anionic phosphates (Fig. 1). Although the electrostatic interactions and steric bulk of Arg are absent for the Ala substitution (R275A), Ala does not clash with the 3’ and 5’ nucleotides, which may explain the smaller effect on base excision for R275A versus R275L.

Although replacement of Arg\textsuperscript{275} with Leu gives a modest 3-fold reduction in G-U activity, it causes a much larger 30-fold reduction in G-T activity (Table 1). It is also observed that Arg\textsuperscript{275} is strictly conserved in TDG enzymes from vertebrate species, for which the biological substrate is likely a G-T mispair in a CpG site, whereas Leu is strictly conserved in the nucleotide flipping position of MUG enzymes, which act upon G-U but have essentially no G-T activity. Together, these observations indicate that an Arg in the nucleotide flipping position is necessary for the efficient cleavage of dT. Perhaps the extended length and/or DNA backbone contacts provided by the Arg side chain are needed to stabilize nucleotide flipping and increase the active site lifetime of dT, which is bulkier and more resistant to cleavage than dU.

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