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Defining the Role of Nucleotide Flipping in Enzyme Specificity using ^{19}F NMR

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Abstract

A broad range of proteins employ nucleotide flipping to recognize specific sites in nucleic acids, including DNA glycosylases, which remove modified nucleobases to initiate base excision repair. Deamination, a pervasive mode of damage, typically generates lesions that are recognized by glycosylases as being foreign to DNA. However, deamination of 5-methylcytosine (mC) generates thymine, a canonical DNA base, presenting a challenge for damage recognition. Nevertheless, repair of mC deamination is important because the resulting G·T mispairs cause C→T transition mutations, and mC is abundant in all three domains of life. Countering this threat are three types of glycosylases that excise thymine from G·T mispairs, including thymine DNA glycosylase (TDG). These enzymes must minimize excision of thymine that is not generated by mC deamination, in A·T pairs and in polymerase-generated G·T mispairs. TDG preferentially removes thymine from DNA contexts in which cytosine methylation is prevalent, including CG and one non-CG site. This remarkable context specificity could be attained through modulation of nucleotide flipping, a reversible step that precedes base excision. We tested this idea using fluorine NMR and DNA containing 2′-fluoro-substituted nucleotides. We find that dT nucleotide flipping depends on DNA context and is efficient only in contexts known to feature cytosine methylation. We also show that a conserved Ala residue limits thymine excision by hindering nucleotide flipping. A linear free energy correlation reveals that TDG attains context specificity for thymine excision through modulation of nucleotide flipping. Our results provide a framework for characterizing nucleotide flipping in nucleic acids using ^{19}F NMR.

Graphical Abstract

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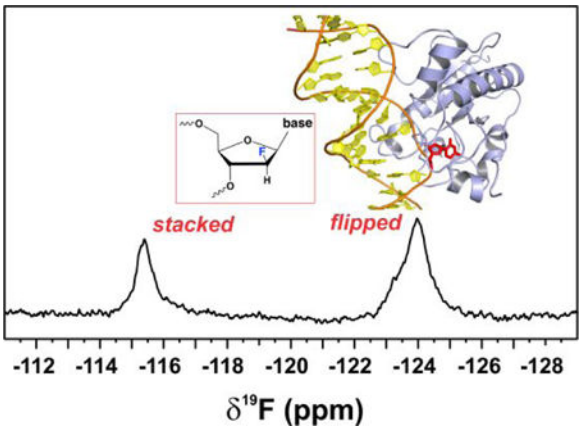
ASSOCIATED CONTENT

Supporting Information

Supplementary Figures S1-S4, Supplementary Tables S1-S4.

Notes

The authors declare no competing financial interest.



INTRODUCTION

Nucleotide flipping is employed by a broad range of proteins to bind specific sites of nucleic acids. Also known as base flipping, this reversible conformational change involves the rotation of one or more nucleotides, by up to 180 degrees, out of the helical stack and into a protein cavity or enzyme active site.[1–2] Nucleotide flipping is used by many different types of enzymes that act on DNA or RNA, including restriction endonucleases[3] and DNA-modifying enzymes such as cytosine methyltransferases, which convert cytosine to 5-methylcytosine (mC).[4] In addition to such "writers" of DNA modifications, nucleotide flipping is employed by many proteins and enzymes that "read" or "erase" DNA modifications.[5] Many DNA repair proteins employ nucleotide flipping, including DNA glycosylases and other factors in base excision repair (BER),[6–7] the Rad4/XPC protein in nucleotide excision repair,[8] and enzymes that perform direct DNA repair such as photolyases and O^6-alkylguanine DNA alkyltransferases.[9–11]

DNA glycosylases use nucleotide flipping to find and remove modified bases from DNA, thereby initiating BER. Deamination is a pervasive type of damage leading to lesions that are processed by DNA glycosylases. While deamination of guanine, adenine, or cytosine generates a lesion that is clearly foreign to DNA, deamination of 5-methylcytosine (mC) produces thymine, one of the four canonical DNA bases. This spontaneous event is mutagenic, because it converts a normal G·mC base pair to a G·T mismatch, a lesion that can generate C→T transitions upon processing by a DNA polymerase.[12–13] Protecting against the threat posed by mC deamination are three types of DNA glycosylases that excise thymine from G·T mispairs, one of which is represented by human thymine DNA glycosylase (TDG),[14–15] the focus of this work.

Among DNA glycosylases, the G·T mismatch enzymes face a particular challenge in selectively removing the rare thymine bases that arise through mC deamination while not acting on the vast background of "normal" thymine. This is not simply a matter of distinguishing between A·T pairs and G·T mispairs, which is itself a non-trivial and poorly understood feat. Rather, G·T mismatch glycosylases must also avoid acting on G·T mispairs that are generated erroneously by DNA polymerases, because faithful repair of polymerase errors must be directed at the misincorporated nucleotide, which can be dG or dT. Indeed,

processing of polymerase-generated G·T mispairs by a mismatch glycosylase could lead to A→G transition mutations, if dG (rather than dT) was incorporated by the polymerase. The question of how G·T mismatch glycosylases attain the specificity to excise thymine arising from mC deamination remains a fundamental problem. It is a question of broad significance given that cytosine methylation is the most abundant DNA modification in the three domains of life, serving as an epigenetic mark in animals and plants and functioning in restriction modification systems of archaea and bacteria.[16]

In mammals, cytosine methylation occurs predominantly at palindromic CG (or CpG) dinucleotides, producing mCG,[17] and mammalian TDG efficiently removes thymine from DNA contexts that are consistent with deamination at mCG sites.[18–21] Cytosine methylation also occurs at some non-CG sites, giving mCH or mCHH (where H = G, A, T, C).[22] Non-CG methylation, including mCAC and mCAG, is found in embryonic stem cells, induced pluripotent stem cells, oocytes, and neurons of the adult brain.[23–25] However, the ability of TDG to protect against mC deamination in most mCHH contexts remains unknown. As such, we used single turnover kinetics experiments to define the activity of TDG for excising thymine from DNA that represents the result of mC deamination in 16 different mCHH contexts. We also investigated a potential role in context specificity for a conserved Gln residue, which forms contacts with bases located on the 3′ side of the flipped nucleotide (dU) in crystal structures of TDG-DNA complexes.[26–27]

We also sought to determine the mechanism by which TDG attains its remarkable context specificity for thymine excision. Prior studies and results here obtained from single turnover kinetics experiments indicate that specificity could be attained at two steps of the TDG reaction, nucleotide flipping or the subsequent chemical step (base excision). However, efforts to discriminate between these possibilities has been impeded in part by the lack of a robust method to directly monitor dT nucleotide flipping for TDG. Here, we describe an approach to characterize nucleotide flipping using fluorine (^{19}F) NMR and DNA containing a single 2′-fluoro-substituted deoxynucleotide. The ^{19}F NMR spectra provide the relative population of nucleotide in the stacked and flipped states, defining the equilibria and informing on the exchange rate for this reversible conformational change. Using ^{19}F NMR, we characterized nucleotide flipping and defined its role in context-dependent thymine excision by TDG. ^{19}F NMR was also used to directly test the possibility that a conserved Ala residue suppresses thymine excision by hindering the flipping of dT nucleotides into the TDG active site, as suggested by previous structural and biochemical studies.[28] Our results provide a general framework for using ^{19}F NMR to characterize nucleotide flipping in free or protein-bound nucleic acids.

RESULTS

Experimental Considerations.

The studies presented here were performed using a construct of human TDG that includes residues 82–308 (TDG$^{82-308}$), which exhibits substrate binding affinity and glycosylase activity that is equivalent to full length TDG (410 residues) for G·T mispairs and other substrates.[26–27] The greater thermal stability and smaller size of TDG$^{82-308}$ (26 kDa) relative to TDG (46 kDa) was beneficial for collecting single turnover experiments at

physiological temperature (37 °C) and enhancing the signal in ^{19}F NMR spectra. TDG activity was defined using single turnover kinetics experiments performed under saturating TDG conditions. The resulting rate constants reflect the maximal rate of product formation ($k_{obs} \approx k_{max}$) and report on steps that include the chemical step and any preceding conformational changes that occur after the initial association of enzyme and substrate, such as nucleotide flipping (Figure 1).[29] This avoids complications arising from characterization of TDG activity using multiple-turnover kinetics experiments, which give steady-state rate constants that are dominated by steps after chemistry, due to slow release of the abasic DNA product and potent product inhibition.[30–31]

Effect of 3′-neighboring bases on thymine excision.

We sought to determine the capacity of TDG to protect against mC deamination when cytosine methylation occurs at CG and non-CG sites. There are 16 potential contexts for cytosine methylation when the two 3′-neighboring bases (+1, +2) can vary, and mC deamination would give 16 contexts for a G·T mispair. The nomenclature used here for DNA containing a G·T mispair in different contexts is "G·Txy", where x and y are the 3′ bases at the +1 and +2 sites, respectively, relative to the mismatched T (Figure 2). For DNA in which only the base at +1 is varied, the nomenclature is G·Tx (or G·Ux).

Performing single turnover kinetics experiments with 16 G·Txy substrates, we find that thymine excision by TDG is highest for substrates with G at the +1 site (Figure 3, Supporting Information Table S1, Figure S1). Moreover, the dependence of activity on the +1 base gives an overall trend that is independent of the base at the +2 site, where G·TGy > G·TAy > G·TCy > G·TTy, for any given base (y) at +2. This trend is in agreement with previous studies which examined four G·Tx substrates in which only the base at the +1 site was varied.[18,21,32] We find a vast 300-fold difference in activity between substrates that are the most and least efficient (G·TGG, G·TTC), revealing a striking degree of context specificity for a DNA glycosylase. Our results underscore the high specificity of TDG for excising thymine from DNA contexts for which cytosine methylation is most prevalent, including CG sites, as indicated by robust activity for each of four G·TGy substrates.

Our results reveal that TDG thymine excision is also modulated by the +2 base, albeit to a lesser extent than for the +1 base (Figure 3). For any given base at +1, the highest thymine excision activity is observed for substrates with G at the +2 site. We note that G·TAG is a remarkably good substrate, much better than the three other G·TAy substrates and nearly as good as the four G·TGy substrates. This is significant because CAG is a prevalent site of non-CG cytosine methylation.[23–25] Thymine is the second most preferred base at the +2 site, consistent with structural findings that TDG contacts a thymine base at the +2 site, via the Gln278 side chain, which also contacts guanine at the +1 site (Figure 4).[26–27]

Role of Gln278 in DNA-context specificity.

To investigate the role of Gln278 in the context specificity of TDG, we determined the thymine excision activity of Q278A-TDG using four G·Tx substrates (Figure 5, Supporting Information Table S2, Figure S2). TDG specificity for the 3′ +1 base is diminished by the Q278A mutation, as evidenced by reduced activity for the optimal G·TG substrate and

enhanced activity for two of the three other G·Tx substrates. Moreover, the difference in activity between the best and worst substrates is 65-fold for TDG and only 14-fold for Q278A-TDG. Still, the mutation does not alter the qualitative trend in activity imparted by the +1 base. Our finding suggests that TDG specificity for a guanine at the +1 site depends on factors in addition to Gln278, which might include a contact from the backbone nitrogen of Ala277 (Figure 4).[26–27] Notably, the glycosylase domain of MBD4 does not contact bases at the +1 or +2 sites, even as thymine excision is modestly dependent on the +1 base (k_{max} is reduced by <3-fold for G·TA, G·TC, and G·TT relative to G·TG).[33] This raises the possibility of inherent differences in the "reactivity" of G·Tx substrates, due perhaps to differential effects of the +1 base on dT flipping.

Does Ala145 impact TDG context specificity?

Previous structural and biochemical studies suggested that Ala145, which is strictly conserved in vertebrate TDG, hinders the flipping of dT nucleotides through a steric clash involving its methyl group with that of dT (Figure 4).[28] This idea was supported by findings that the A145G mutation causes a large (13-fold) increase in glycosylase activity for G·T substrates but has no effect on activity for G·U substrates (U lacks the methyl of T).[28] Another study showed that the impact of DNA context (3′ +1 base) on uracil excision depends on the size of substituents at C5 of uracil (Figure 4).[21] For example, the context effect is much greater for excision of 5-BrU relative to 5-FU (both paired with G). Those prior results are supported by findings here that the DNA context has a much greater impact on excision of T relative to U; activity varies by 65-fold for G·Tx substrates compared to 12-fold for G·Ux (Figure 5, Supporting Information Table S2). These previous findings prompted the question of whether the A145G mutation might reduce the strong dependence of thymine excision on the +1 base (by relieving the steric hindrance to dT flipping). On the contrary, we find that the A145G mutation increases thymine excision activity dramatically but uniformly, by 13-fold for all four G·Tx substrates (Figure 5). Thus, while removing the methyl of T reduces the DNA context specificity of base excision, the same result is not obtained by removing the methyl of Ala145.

Limitations in the results from single turnover kinetics.

Rate constants (k_{max}) obtained from single turnover experiments can be influenced by multiple steps of the TDG reaction, including nucleotide flipping, the chemical step(s), and any post-flipping conformational changes that may lead to a productive E·S complex (Figure 1). An experimental approach to directly monitor the fraction of nucleotide in the flipped versus stacked conformations could potentially reveal which step of the TDG reaction confers its stringent context specificity for thymine excision. While fluorescent base analogs such as 2-aminopurine are used widely to monitor nucleotide flipping,[34] these probes are typically indirect since they are usually positioned adjacent to the nucleotide that is flipped. Moreover, the data can be complicated to interpret and often do not readily give the relative population of the nucleotide in the stacked and flipped conformations (K_{flip}) for protein-bound DNA. To address this problem, we developed an approach to characterize nucleotide flipping using fluorine NMR.

Characterizing nucleotide flipping by fluorine NMR.

^{19}F NMR is a powerful approach for studying structure and dynamics in biological systems.[35] Its power stems from the absence of fluorine in most biomolecules, the 100% natural abundance of ^{19}F, and the hypersensitivity of ^{19}F to its local environment, where the range of chemical shifts is up to 100-fold greater than that observed for corresponding ^{1}H nuclei. Our approach for monitoring nucleotide flipping involves simple 1D NMR experiments, using DNA in which ^{19}F replaces ^{1}H at the 2′ position of a nucleotide. 2′-F-substituted nucleotides are synthesized in two forms, 2′-F-ribo (α) and 2′-F-arabino (β) (Chart 1). The sugar pucker of 2′-F-α nucleotides is compatible with A-form DNA or RNA, while that of 2′-F-β nucleotides is compatible with B-form DNA.[26, 36] Our studies employ the 2′-F-β forms exclusively, including 2′-F-β-dT and 2′-F-β-dU, hereafter referred to as dT$^{2'F}$ and dU$^{2'F}$.

Importantly, 2′-F-β-substituted deoxynucleotides have been used widely in structural and biochemical studies of DNA glycosylases, because the 2′-F substitution precludes hydrolysis of the *N*-glycosyl bond due to transition-state destabilization.[37–40] This enables formation of a stable enzyme-substrate complex, where the 2′-F nucleotide flips into the active site in the absence of bond cleavage, as demonstrated for TDG and many other DNA glycosylases.[26–27, 41–43] However, to our knowledge, 2′-F-substituted nucleic acids have not been used for ^{19}F NMR studies of nucleotide flipping. We reasoned that the chemical environment of the fluorine reporter would likely differ substantially for the stacked and flipped conformations of dT$^{2'F}$ (or dU$^{2'F}$), leading to distinct ^{19}F chemical shifts. The studies below confirm this idea and show that dT$^{2'F}$ and dU$^{2'F}$ are excellent probes of nucleotide flipping using ^{19}F NMR.

We illustrate the ^{19}F NMR method for studying nucleotide flipping through studies of DNA containing dT$^{2'F}$ and dU$^{2'F}$ (Figure 6). We consider initially the spectrum for free DNA containing dT$^{2'F}$ in an A·T base pair, which exhibits a single peak. This is consistent with the expectation that, although A·T pairs exhibit rapid opening and closing, the nucleotides are predominantly stacked in the DNA duplex, as shown by NMR imino exchange studies.[36, 44–45] The spectrum for TDG-bound A·T DNA exhibits a single peak at the same chemical shift but with increased linewidth relative to the peak for free DNA. While TDG does not efficiently remove T from A·T pairs it has relatively high affinity for nonspecific DNA, binding with a dissociation constant (K_d <0.3 μM) that is at least 1000-fold below the TDG concentration used in the NMR sample.[40, 46] As such, the NMR results are most reasonably explained by nonspecific binding of TDG to the A·T DNA, increasing its overall rotational correlation time and increasing the ^{19}F resonance linewidth for dT$^{2'F}$ while not substantially altering its chemical environment. The peak broadening could also reflect binding of TDG to multiple sites on the DNA, which may cause minor changes to the environment of dT$^{2'F}$. The absence of a substantial chemical shift difference for dT$^{2'F}$ in free versus TDG-bound A·T DNA suggests that TDG does not stably flip dT from A·T pairs. While novel, this finding is not unexpected given that TDG does not efficiently excise thymine from A·T pairs.

We next consider NMR spectra for free and TDG-bound DNA that contains dT$^{2'F}$ in a G·T mispair (G·TGG; Figure 6). The spectrum for dT$^{2'F}$ in free DNA features a single peak,

consistent with the expectation that nucleotides in a G·T mispair, although kinetically and thermodynamically destabilized relative to Watson-Crick pairs, are still predominantly stacked in the DNA duplex ($K = 7 \times 10^{-4}$ for G·T opening), as shown by NMR imino exchange studies.[47] The spectrum for TDG-bound G·T DNA exhibits two broad peaks with a large difference in chemical shift ($\Delta\delta^{19}$F of 8.6 ppm), indicating that the $dT^{2'F}$ nucleotide partitions between two distinct chemical environments. The downfield peak is attributed to the stacked conformation of $dT^{2'F}$ for TDG-bound DNA because it has the same δ^{19}F as the peak for free DNA; the upfield peak (δ^{19}F −124 ppm) is attributed to $dT^{2'F}$ flipped into the TDG active site. Notably, assignment of these peaks to the stacked and flipped states of $dT^{2'F}$ for TDG-bound DNA are supported by the other NMR results described below. The possibility that the NMR sample contains a substantial amount of free DNA is excluded by the presence of TDG at a concentration that is at least 10^4-fold above the K_d for its binding to G·T DNA.[26, 40] Thus, the ^{19}F NMR spectra reveal that a substantial fraction of the mismatched dT nucleotide remains stacked for TDG-bound DNA, even for a G·T mispair in the context that gives maximal thymine excision activity (G·TGG). Similar findings are provided below for G·T mispairs in other DNA contexts.

The spectrum for G·T DNA bound to A145G-TDG features only one significant peak, which resonates slightly upfield from the peak for flipped $dT^{2'F}$ for the same DNA bound to wild-type TDG. This observation and additional findings below support the idea that Ala145 hinders flipping of dT into the TDG active site (Figure 4). Notably, peaks for both the stacked and flipped states of $dT^{2'F}$ are observed in spectra for other G·T DNA bound to A145G-TDG, as shown below (Figure 6).

The ^{19}F NMR spectrum for DNA containing $dU^{2'F}$ in a G·U pair features a single peak (Figure 6), consistent again with the expectation that the nucleotides are predominantly stacked in the duplex, based on NMR imino exchange studies for G·T mispairs (see above).[47] The spectrum for TDG-bound G·U DNA exhibits no peak at the expected δ^{19}F for stacked $dU^{2'F}$ but has two broad and overlapping peaks located about 6 ppm upfield of the peak for TDG-free DNA. Together, the spectra indicate that for TDG-bound G·U DNA, the vast majority of dU is flipped, with no evidence for a substantial population of stacked dU. Observation of two overlapping peaks suggests multiple conformations for the flipped state of $dU^{2'F}$, which is notable because a single conformation for flipped $dU^{2'F}$ is observed in a high-resolution (1.54 Å) crystal structure of the identical complex (TDG^{82-308}, G·U DNA; Figure 4).[26] These observations suggest that one of the conformations indicated by ^{19}F NMR predominates in the crystal state. We also collected ^{19}F NMR data for G·U DNA bound to a smaller TDG construct ($TDG^{111-308}$) that has 30 fewer N-terminal residues and weaker substrate binding relative to the larger TDG^{82-308} construct.[26] The spectrum exhibits a single though broad peak for $dU^{2'F}$, suggesting multiple conformations for the flipped state and faster conformational exchange relative to TDG^{82-308}.

Equilibria and dynamics of nucleotide flipping.

The ^{19}F NMR spectra provide the equilibrium constant for reversible nucleotide flipping (K_{flip}), from the ratio of integrals for peaks corresponding to the flipped (I_F) and stacked (I_S) states ($K_{flip} = I_F/I_S$). For example, the spectrum for G·TGG DNA bound to TDG (Figure 6)

yields K_{flip} of 2.1, while a K_{flip} of 30 is obtained for the same DNA bound to A145G-TDG. An upper or lower limit for K_{flip} can be assigned in the absence of a significant peak for the flipped or stacked state, respectively.

The ^{19}F NMR spectra also inform the dynamics of nucleotide flipping. Observation of well resolved peaks for the stacked and flipped conformations of dT$^{2'F}$ indicates that the conformational exchange rate (k_{ex}) for these two states is slow relative to the difference in resonance frequency ($\Delta\nu$) for the two peaks (that is, slow on the NMR timescale).[48] As such, the NMR spectra yield an upper limit for k_{ex} (eq. 1).

$$k_{ex} < (0.71\pi)|\Delta\nu| \quad (1)$$

Considering the spectrum for G·TGG DNA bound to TDG (Figure 6), the difference in resonance frequency for peaks representing the stacked and flipped conformations of dT$^{2'F}$ ($\Delta\nu$ = 4043 Hz; $\Delta\delta^{19}$F = 8.6 ppm) gives k_{ex} <9000 s^{-1}. Similarly, NMR spectra for G·U DNA, free and TDG-bound, suggest that k_{ex} for dU flipping is well below 6800 s^{-1} (assuming δ^{19}F for the stacked conformation of dU is equivalent for free and TDG-bound G·U DNA). As noted above, NMR spectra for G·U DNA bound to TDG$^{82-308}$ reveal two overlapping peaks, indicating multiple conformations for flipped dU$^{2'F}$ in slow exchange; the difference in resonance frequency ($\Delta\nu$ ~431 Hz) indicates k_{ex} < 960 s^{-1}. Observation of a broader peak for G·U DNA bound to TDG$^{111-308}$, with a chemical shift at about the weighted average of the two peaks for TDG$^{82-308}$, suggests flipped dU samples multiple conformations in fast exchange. Notably, structures of the two TDG constructs (TDG$^{82-308}$, TDG$^{111-308}$) bound to G·U DNA reveal differences in the conformation of a catalytic loop (residues 192–204),[26] among other moieties, which might account for differences in the dynamics of flipped dU indicated here by ^{19}F NMR.

Dependence of nucleotide flipping on DNA context.

Using this ^{19}F NMR approach we sought to determine the dependence of dT flipping (from a G·T mispair) on the 3′-neighboring bases, examining the four potential bases at +1 and some at the +2 site, for TDG and A145G-TDG (Figure 7), giving a representative look at the 16 G·Txy substrates examined in the activity assays. As noted above, the downfield peaks (near −115.5 ppm) report on the stacked conformation of dT$^{2'F}$ and the upfield peaks (near –124 ppm) report on the flipped state. Results for TDG indicate that a large fraction of dT$^{2'F}$ is flipped for G·TGG and G·TGT while no substantial flipping is observed for G·TAT or G·TTT DNA. Notably, a much higher fraction of dT$^{2'F}$ is flipped for G·TAG relative to G·TAT, demonstrating that the +2 base can impact flipping substantially. Flipping for G·TAG is relevant because CAG is a prominent site of non-CG methylation. Notably, the dependence of nucleotide flipping on DNA context exhibits a trend (G·TGG > G·TGT > G·TAG > G·TAT > G·TTT) that is identical to that observed for the dependence of thymine excision activity (k_{max}) on DNA context (Figure 3).

Remarkably, for any given G·Txy DNA, the fraction of dT$^{2'F}$ flipped is much greater for A145G-TDG relative to TDG (Figure 7). Indeed, the vast majority of dT$^{2'F}$ is flipped for

G·TGG or G·TGT bound to A145G-TDG, and the fraction $dT^{2'F}$ flipped is relatively high for DNAs contexts that exhibit little or no flipping for wild-type TDG, including G·TAT and G·TTT. This provides the first direct evidence that Ala145 hinders dT flipping, an idea that had been suggested by previous studies.[28] As observed for wild-type TDG, we find a strong qualitative correlation between the fraction of dT flipped and thymine excision activity for A145G-TDG.

Linear dependence of glycosylase activity (k_{max}) on K_{flip}.

The similar DNA-context dependence observed for dT flipping and thymine excision suggested that a quantitative analysis could be informative. Indeed, we find a linear correlation between log k_{max} and log K_{flip} for TDG and for A145G-TDG (Figure 8, Supporting Information Table S3, Figure S3). We note that k_{max} values used in this analysis were obtained from enzyme activity assays performed at the same temperature as the NMR experiments (18 °C).

For TDG, the linear free energy (LFE) correlation has a slope of 1.03 (r = 0.996), indicating that regulation of thymine excision activity by DNA context (3′ bases) is derived through modulation of nucleotide flipping. The results for A145G-TDG reveal a similar LFE correlation, and further analysis suggests that for at least one DNA context, regulation of thymine excision by 3′ bases involves perturbation of a post-flipping step, in addition to modulation of nucleotide flipping. Thus, a fitting that includes all data for A145G-TDG gives a slope of m = 1.20 (r = 0.949; dotted red line) and appears to be skewed by G·TTT (red star). By contrast, fitting that excludes G·TTT gives an improved correlation, with a slope of m = 0.97 (r = 0.988; solid red line). Notably, k_{max} for G·TTT is ~6-fold lower than predicted by the latter LFE correlation, suggesting that for substrates with thymine at the +1 site (G·TT), thymine excision could be suppressed by a mechanism that acts not only on dT flipping but also on a post-flipping step, which could include a conformational change needed to give a productive E·S complex or the chemical step(s). Notably, fitting for wild-type TDG excluded G·TTT due to the absence of a peak for flipped dT in the ^{19}F NMR spectrum (Figure 7).

The possibility that a post-flipping step may be impaired for substrates with T at the +1 site was also investigated using G·U DNA. We collected ^{19}F NMR spectra for TDG-bound DNA that contained $dU^{2'F}$ in one of four G·Ux pairs (Figure 9, Supporting Information Table S4, Figure S4). Remarkably, the NMR spectra indicate that $dU^{2'F}$ is predominantly flipped into the TDG active site, with little evidence for the stacked state, regardless of the +1 base. While the NMR spectra indicate that the fraction of dU flipped is similar for all G·Ux DNA, uracil excision is dramatically reduced for G·UT relative to the three other G·Ux substrates. Indeed, compared to G·UG, k_{max} is reduced by 20-fold for G·UT but by less than 3-fold for G·UA and G·UC. Together, our findings suggest that impairment of T (or U) excision by a thymine at the 3′ +1 site involves effects on both nucleotide flipping and on a post-flipping step.

Temperature dependence of nucleotide flipping.

Previous studies and results here reveal that the temperature dependence of TDG activity is far more pronounced for G·T relative to G·U substrates,[49] particularly for mispairs in a non-CG context. Indeed, the fold increase in G·T activity at 37 °C relative to 18 °C ($k_{max}^{37C}/k_{max}^{18C}$) is 11, 13, 34, and 44 for G·TG, G·TA, G·TC, and G·TT substrates, respectively (Supporting Information Table S4). The corresponding changes are much smaller for G·U substrates, with $k_{max}^{37C}/k_{max}^{18C}$ ratios of 4 to 7 for G·Ux substrates. Notably, the ^{19}F NMR results obtained at 18 °C indicate that for any TDG-bound G·Ux DNA, the $dU^{2'F}$ nucleotide is predominantly flipped, with no substantial peak observed for the stacked state (Figure 9). By contrast, for TDG-bound to the optimal G·TGG DNA, a substantial fraction (~32%) of $dT^{2'F}$ remains stacked (Figure 6). These observations prompted the question of whether the equilibria for dT flipping (K_{flip}) increases with temperature for TDG-bound G·T DNA, which we investigated using ^{19}F NMR. Indeed, the spectra reveal that the fraction of $dT^{2'F}$ nucleotide in the flipped state increases steadily with temperature, with $dT^{2'F}$ predominantly stacked at 5 °C and largely flipped at 34 °C (Figure 10). The robust temperature-dependent increase in the fraction of $dT^{2'F}$ in the flipped conformation provides a reasonable explanation for the more pronounced temperature dependence of TDG glycosylase activity (k_{max}) for G·T relative to G·U substrates. In other words, the contribution of nucleotide flipping to the temperature dependence of k_{max} is greater for G·T relative to G·U substrates.

DISCUSSION

Protection against mC deamination by TDG.

We investigated the capacity of human TDG to protect against mC deamination for 16 possible mCHH contexts, using a series of 16 G·Txy substrates. Our results indicate that TDG thymine excision activity is greatest for G·T mispairs located in a context for which cytosine methylation is most prevalent, including all four CG(H) contexts and CAG, a prominent site for non-CG methylation in embryonic stem cells, induced pluripotent stem cells, and oocytes,[23–25] among other possible cell types. By contrast, TDG activity is reduced by 11- to 22-fold for G·TAC relative to the optimal G·TG(H) substrates, which is notable because CAC is a prominent site of non-CG methylation, particularly in the adult mammalian brain.[23–25] Our finding raises the question of whether TDG offers substantial protection against mC deamination for mCAC sites. Our results will inform the ability of TDG to protect against mC deamination at other non-CG methylation sites that may be identified in future studies.

The DNA-context specificity of TDG could reflect a biological imperative to maximize its capacity to excise “damaged” thymine bases that arise through mC deamination while minimizing activity on undamaged thymine, in A·T base pairs or G·T mispairs generated by a DNA polymerase. Faithful repair of polymerase-generated G·T mispairs must be directed at the newly incorporated nucleotides (nascent strand), as observed for mismatch repair (MMR). TDG processing of polymerase-generated G·T mispairs could generate an A→G transition mutation if it excises thymine from a G·T mispair for which dG was mistakenly incorporated. TDG is degraded in S phase,[50–52] when G·T mispairs generated during bulk DNA replication are expected to arise. Nevertheless, if degradation is incomplete, the

context specificity of thymine excision could minimize the activity of any residual TDG on thymine bases that are not generated by mC deamination, including polymerase-generated G·T mispairs. This specificity could also minimize TDG activity on G·T mispairs generated by polymerases functioning outside of DNA replication, in translesion synthesis, DNA repair, or DNA recombination.

The context specificity of TDG might also serve to minimize aberrant thymine excision from the vast background of A·T pairs. Although TDG activity is very weak for A·T pairs at room temperature (k_{max} = ~1 × 10^{-5} min^{-1}),[21] the activity is likely to be substantially higher at 37 °C. While TDG activity for A·T pairs is too weak to define the effect of DNA context (e.g., 3′ +1 base), the activity is substantial for A·U pairs and, more generally, on A·5xU pairs (x is H, F, Cl, Br).[21] TDG activity for A·5xU pairs depends strongly on sequence context (3′ +1 base), giving a trend (A·5xUG > A·5xUA > A·5xUC > A·5xUT) that is identical to that observed here for G·U and G·T mispairs. Thus, the strong context specificity observed for G·T mispairs is also likely to occur for residual activity on the vast background of A·T pairs. This could serve to limit any residual A·T activity to those in an A·TG context.

The robust specificity of TDG for excising thymine from DNA contexts that are prevalent for cytosine methylation, shown here and in previous studies,[18–21] indicates that TDG has evolved with the capacity to initiate base excision repair of deaminated mC. However, evidence for this activity *in vivo* is somewhat limited, due perhaps to experimental challenges posed by the fact that TDG deficiency causes embryonic lethality (in mice),[53–54] and that another mammalian DNA glycosylase, methyl binding domain IV (MBD4), also excises thymine from G·T mispairs.[55–56] Nevertheless, one study found that depletion of TDG in mammalian cell (MEF) extracts abrogates detectable excision of T from a G·T mispair in DNA, suggesting TDG is the predominant glycosylase acting on deaminated mC (in MEF extracts).[53] In another study, a rectal cancer patient with MMR deficiency exhibited elevated C→T mutations at CpG sites (methylated) in tumor suppressor genes, which was attributed to reduced TDG protein levels in the tumor cells (due to a D284Y mutation).[57] Two studies found that while depletion of MBD4 causes an increase in C→T transitions at CpG sites, the results also suggested that a factor in addition to MBD4 contributes to repair of deaminated mC.[58–59] The other factor is likely to be TDG, since it is the only other mammalian enzyme that has specificity for excising deaminated mC. While these findings collectively suggest that TDG repairs deaminated mC *in vivo*, additional studies are clearly warranted.

Mechanism of context specificity elucidated by ^{19}F NMR.

Among the 16 G·Txy substrates examined here, we find a vast 300-fold difference in activity (k_{max}) between the best and worst (G·TGG and G·TTC). This reveals a strikingly high level of DNA-context specificity for a DNA glycosylase. Addressing the fundamental question of how a DNA glycosylase attains such remarkable context specificity necessitated a new approach to directly monitor nucleotide flipping. We developed such an approach using ^{19}F NMR, a powerful probe of conformational change in biological systems. Our method involves simple 1D NMR experiments and samples that feature a single fluorine atom, using DNA containing a 2′-F-β-substituted nucleotide. These nucleotide analogs are fully

compatible with B form DNA and resistant to *N*-glycosyl bond cleavage by DNA glycosylases. Our ^{19}F NMR results show that 2′-F-dT and -dU are outstanding probes of TDG-mediated nucleotide flipping. The NMR spectra reveal well resolved peaks corresponding to the stacked and flipped conformations of the nucleotide, providing the relative population in each state. As such, the results provide the equilibrium constant for nucleotide flipping and an upper limit for the exchange rate (k_{ex}).

Using this ^{19}F NMR approach we show that dT flipping from a G·T mispair is strongly dependent on 3′-neighboring bases (+1, +2), for TDG and A145G-TDG. Our results provide the first direct evidence that TDG attains its stringent DNA context specificity for thymine excision by modulating the equilibria for dT flipping (K_{flip}). More broadly, our findings constitute the first direct evidence, to our knowledge, that a glycosylase attains specificity for acting on a particular DNA context through modulation of nucleotide flipping (K_{flip}).

We observe a linear free energy (LFE) correlation for the dependence of thymine excision activity (log k_{max}) on dT nucleotide flipping (log K_{flip}) by TDG. The slope of unity indicates that the regulation of thymine excision by DNA context (3′ +1, +2 base) is attained through modulation of nucleotide flipping. The results also indicate that for G·T (or G·U) substrates with thymine at the +1 site (e.g., G·TTy, G·UTy), base excision activity (k_{max}) is reduced by a post-flipping mechanism that acts in addition to effects on nucleotide flipping. This could involve a mechanism that perturbs the formation of a productive enzyme-substrate complex and/or an effect on the chemical step(s) of the reaction.

Ala145 hinders dT flipping.

The results of our ^{19}F NMR studies provide the first direct evidence that Ala145 hinders flipping of dT into the TDG active site, an idea that had been suggested by previous biochemical and structural studies.[28] Indeed, K_{flip} is 9- to 14-fold higher for A145G-TDG relative to TDG for a G·T mispair in several DNA contexts (Supporting Information Table S3). Moreover, thymine excision is much (13-fold) faster for A145G-TDG versus TDG, for G·T mispairs in a CG context and in the three non-CG contexts examined here. Notably, flipping appears to be somewhat more productive for A145G-TDG relative to TDG, as indicated by the observation in the LFE correlations that for a given degree of flipping (K_{flip}), thymine excision activity (k_{max}) is about twofold higher for A145G-TDG. This suggests that Ala145 adversely impacts a step after nucleotide flipping, perhaps by hindering formation of a productive E·S complex or by perturbing the chemical step. These observations are important given that Ala145 is strictly conserved in vertebrate TDG. Previous studies suggest that Ala145 serves to minimize excision of thymine from A·T pairs, [28] even as it greatly reduces TDG activity on G·T mispairs and thus its capacity to protect against mutations caused by mC deamination. As noted previously, the compromised G·T activity of TDG offers a reasonable explanation for the high frequency of C→T transition mutations at CG dinucleotides in cancer and genetic disease.[19, 60–62]

^{19}F NMR to study nucleotide flipping in other proteins.

We anticipate that the ^{19}F NMR studies presented here will provide a general approach to monitor nucleotide flipping by proteins that use this ubiquitous mechanism to bind specific

sites of nucleic acids. Nucleotide flipping is employed by a broad range of proteins that perform a variety of functions and are found in all three domains of life. Two previous studies employed ^{19}F NMR to characterize nucleotide flipping, for a cytosine methyltransferase and uracil DNA glycosylase.[63–64] However, for these studies the ^{19}F substitution was in the nucleobase, which provides a useful probe of flipping but can substantially perturb protein interactions and thereby alter the equilibria for flipping. Our approach, using 2′-F-substituted nucleotides in DNA can, in principle, be employed for any canonical DNA or RNA nucleotide, or analogues thereof. In practice, the utility of our approach for a given system will depend on factors including the amenability of the protein to NMR studies (solubility, etc.), and the availability of a phosphoramidite to synthesize oligonucleotides containing the desired 2′-F-substitution. We note that phosphoramidites for the 2′-F-substituted forms of the four canonical deoxynucleotides in DNA are commercially available and synthesis of phosphoramidites for 2′-F-substituted nucleotides has been described for several modified nucleotides, including 5-formyl-dC, 5-carboxyl-dC, and N7-methyl-dG.[27, 65–66]

MATERIALS AND METHODS

Materials.

TDG$^{82-308}$ was expressed and purified as described.[26] The expression vector for A145G-TDG$^{82-308}$ was generated via site-directed mutagenesis using the Quickchange II system (Agilent Technologies), as described,[67] and the expression vector for Q278A-TDG$^{82-308}$ was obtained from ATUM (Newark, CA). The variant enzymes were expressed and purified as described for wild-type TDG$^{82-308}$.[26] Purity of the enzymes was >99% as assessed by SDS-PAGE with Coomassie staining. Enzyme concentration was determined by absorbance at 280 nm,[40, 68] using an extinction coefficient of $\varepsilon^{280} = 17.4\ mM^{-1}cm^{-1}$ (for TDG$^{82-308}$ and its variants).[69]

Oligodeoxynucleotides (ODNs) were obtained from IDT or the Keck Foundation Biotechnology Resource Laboratory of Yale University. ODNs containing the 2′-fluoroarabino analogs of deoxyuridine or deoxythymidine were synthesized at Yale using phosphoramidites obtained from Glen Research or Link Technologies.[67] TDG binds productively to DNA containing these analogs, which are fully resistant to *N*-glycosyl bond cleavage because the single-atom fluorine substitution destabilizes the chemical transition-state of the reaction.[37, 40–41, 67] ODNs were purified by reverse phase HPLC using an XBridge OST C18 column (Waters Corp.), mobile phases that contained 0.1 M TEAA pH 7.0 and either 5% (A) or 15% (B) acetonitrile, a flow rate of 3.5 ml/min, and a gradient of 35–60% B over 19 min.[70] Purified ODNs were dried in a vacuum concentrator and exchanged into 0.01 M Tris–HCl pH 8, 0.05 M NaCl, 1 mM EDTA; the ODN concentration was determined by absorbance.[21] The 28 bp duplex DNA was made by mixing a target strand (5′-ACCAGTCCATCGCTCA XxyACAGAGCTG; X = T, U, dT$^{2'F}$, or dU$^{2'F}$, x and y are any of the four DNA bases) and its complement, heating to 80 °C, and slowly cooling to room temperature.

Glycosylase assays.

Glycosylase activity was monitored using single turnover kinetics experiments performed under saturating enzyme conditions ([E] > [S], [E] >> K_d), giving rate constants that are not impacted by enzyme-substrate association or by product release or product inhibition and thereby reflect the maximal rate of product formation ($k_{obs} \approx k_{max}$).[29] Reactions were initiated by adding enzyme to DNA substrate (0.5 μM) in HEN.1 buffer (0.02 M HEPES pH 7.5, 0.1 M NaCl, 0.2 mM EDTA), at 37 °C (unless noted otherwise). Aliquots were removed and added to 50% (v:v) quench solution (0.3 M NaOH, 0.03 M EDTA) to immediately halt the reaction, and samples were heated for 5 min at 85 °C to quantitatively cleave the DNA backbone at abasic sites. The resulting DNA fragments were resolved by HPLC and peak integrals were used to determine fraction product.[21, 29] Rate constants were determined from fitting progress curves (fraction product versus time) to eq. 2 using non-linear regression:

$$\text{fraction product} = A\left(1 - \exp\left(-k_{obs}t\right)\right) \quad (2)$$

where A is the amplitude, k_{obs} is the rate constant, and t is reaction time. The presence of saturating enzyme ([E] >> K_d) in the single turnover experiments was confirmed by observation that the rate constants (k_{obs}) were the same (within experimental error) for multiple enzyme concentrations (typically, 1 μM, 2 μM). Previous studies show that TDG and TDG$^{82-308}$ bind tightly to G·T DNA substrates (K_d < 0.02 μM).[26, 40]

^{19}F NMR Spectroscopy.

Fluorine NMR experiments were performed on a Varian 500 MHz spectrometer (470.13 MHz for ^{19}F) equipped with four channels, a Z-axis gradient, and a 5 mm HFCN probe (optimized for ^{1}H, ^{19}F, ^{13}C and ^{15}N). ^{19}F NMR experiments were collected with 8192 complex points, an acquisition time of 0.66 s, a relaxation delay of 1.0 s, a carrier frequency of −119 ppm relative to δ^{19}F for trifluoroacetic acid (TFA), and at 18 °C (unless stated otherwise). ^{19}F NMR spectra were collected with 1000–2000 scans for free DNA and 7,700–25,000 scans for protein-DNA complexes. The data were processed by applying exponential multiplication with 25 Hz line broadening prior to Fourier transformation, using NMRpipe.[71] The observed ^{19}F chemical shifts (δ^{19}F) are relative to TFA (external). The samples contained ~0.2 mM DNA and 0.3 mM enzyme (TDG$^{82-308}$ or A145G-TDG$^{82-308}$). Enzyme-free samples contained 0.05–0.1 mM DNA. All samples were in a buffer consisting of 15 mM Tris-HCl pH 7.5, 0.1 M NaCl, 10% D_2O.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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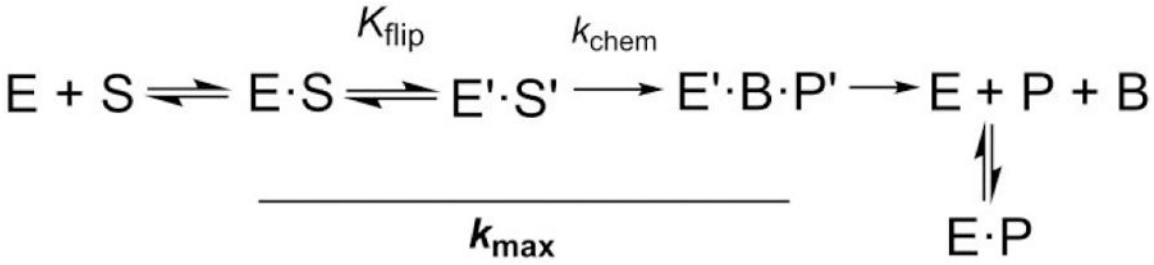


Figure 1.

Minimal kinetic mechanism for the TDG reaction. Association of enzyme (E) and DNA substrate (S) gives an initial collision complex (E·S), then nucleotide flipping (K_{flip}) and potentially other conformational changes give the reactive enzyme-substrate complex (E′·S′). In the chemical step (k_{chem}), cleavage of the *N*-glycosyl bond and addition of the nucleophile (water) generate the ternary product complex (E′·B·P′, where B is the excised base and P is abasic DNA). Dissociation of E′·B·P′ likely involves rapid release of B and slow release of abasic DNA. The solid line denotes reaction steps that contribute to k_{max}, the rate constant obtained from the single turnover kinetics experiments.

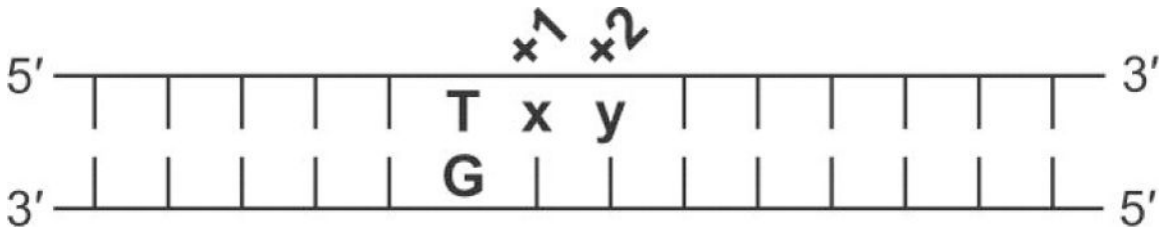


Figure 2.

DNA nomenclature. DNA that contains a G·T mispair is referred to as "G·Txy", where x and y represent bases at the +1 and +2 sites, respectively, relative to the mismatched T. For example, "G·TGT" has G at the +1 and T at the +2 site. DNA for which only the +1 base varies is denoted as G·Tx (or G·Ux). The full DNA sequence is given in Materials and Methods.

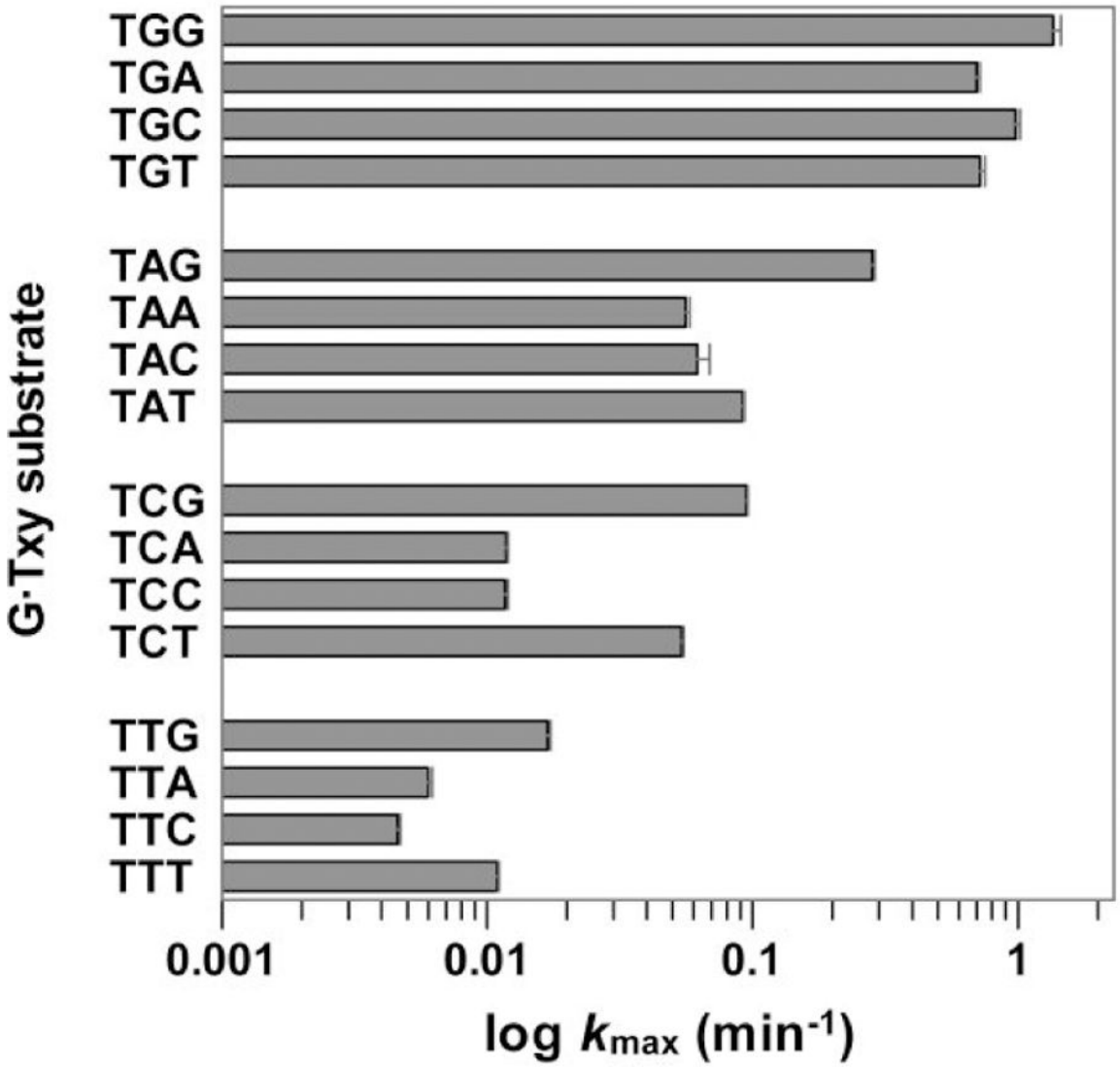


Figure 3.

TDG thymine excision activity (log k_{max}) for G·Txy substrates (abbreviated as Txy) at 37 °C. Substrates are clustered in groups of four, according to the base at +1. Data fitting and k_{max} values are given in Supporting Information Figure S1, Table S1.

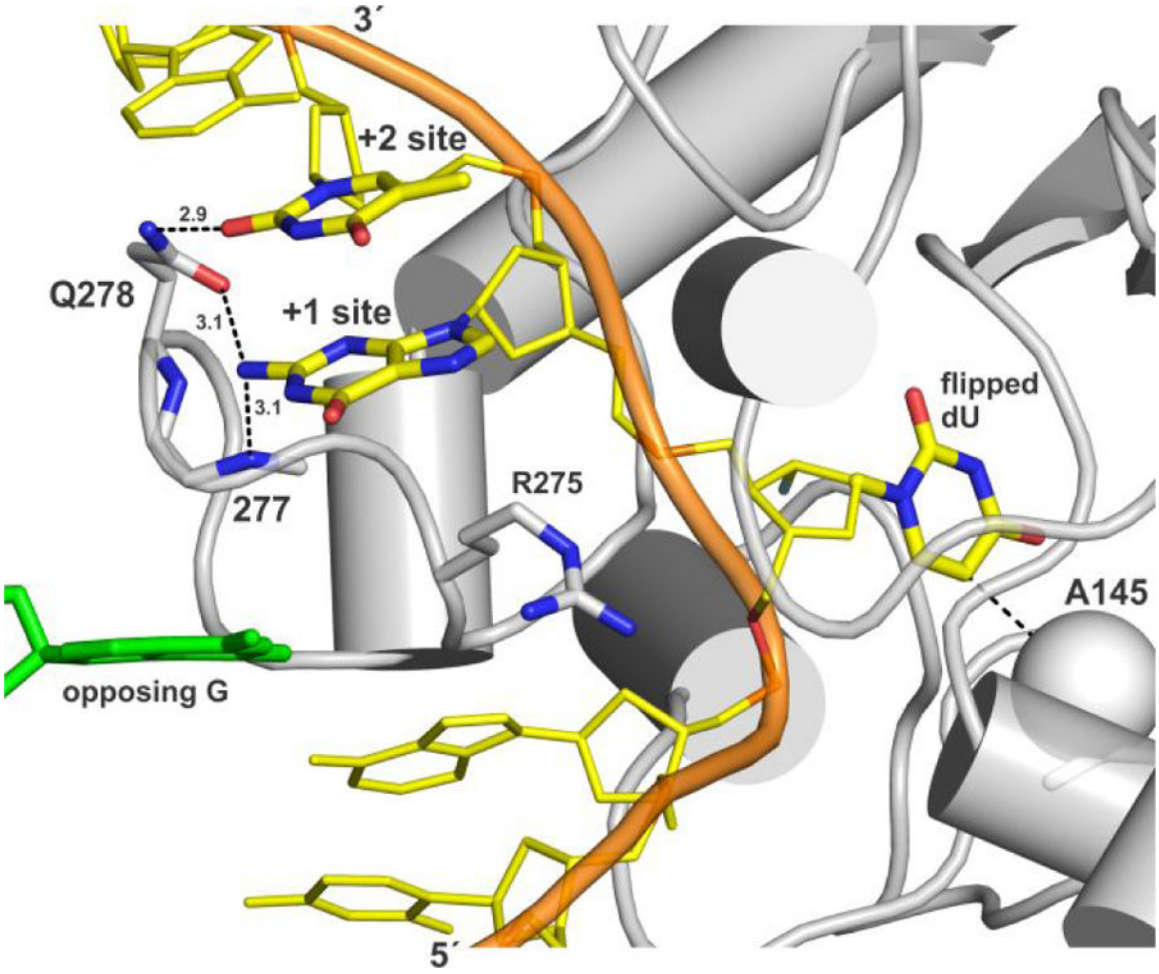


Figure 4.

Structure of TDG^{82-308} bound to DNA containing a G·U mispair (PDB ID: 5HF7). Interactions with Gua at the +1 site and Thy at +2 site involve the Q278 side chain and the backbone amide of A277. The dU nucleotide is flipped into the active site and the guanine that it had been paired with (opposing base) is in green. The methyl group of A145 is shown as a sphere, and the dotted line connects this group with C5 of flipped dU (methyl of T is located at C5). No structure has been reported for TDG with dT flipped into the active. Previous findings and results here support a model whereby flipping of dT is partially hindered by a clash between its methyl and that of A145 (see text).

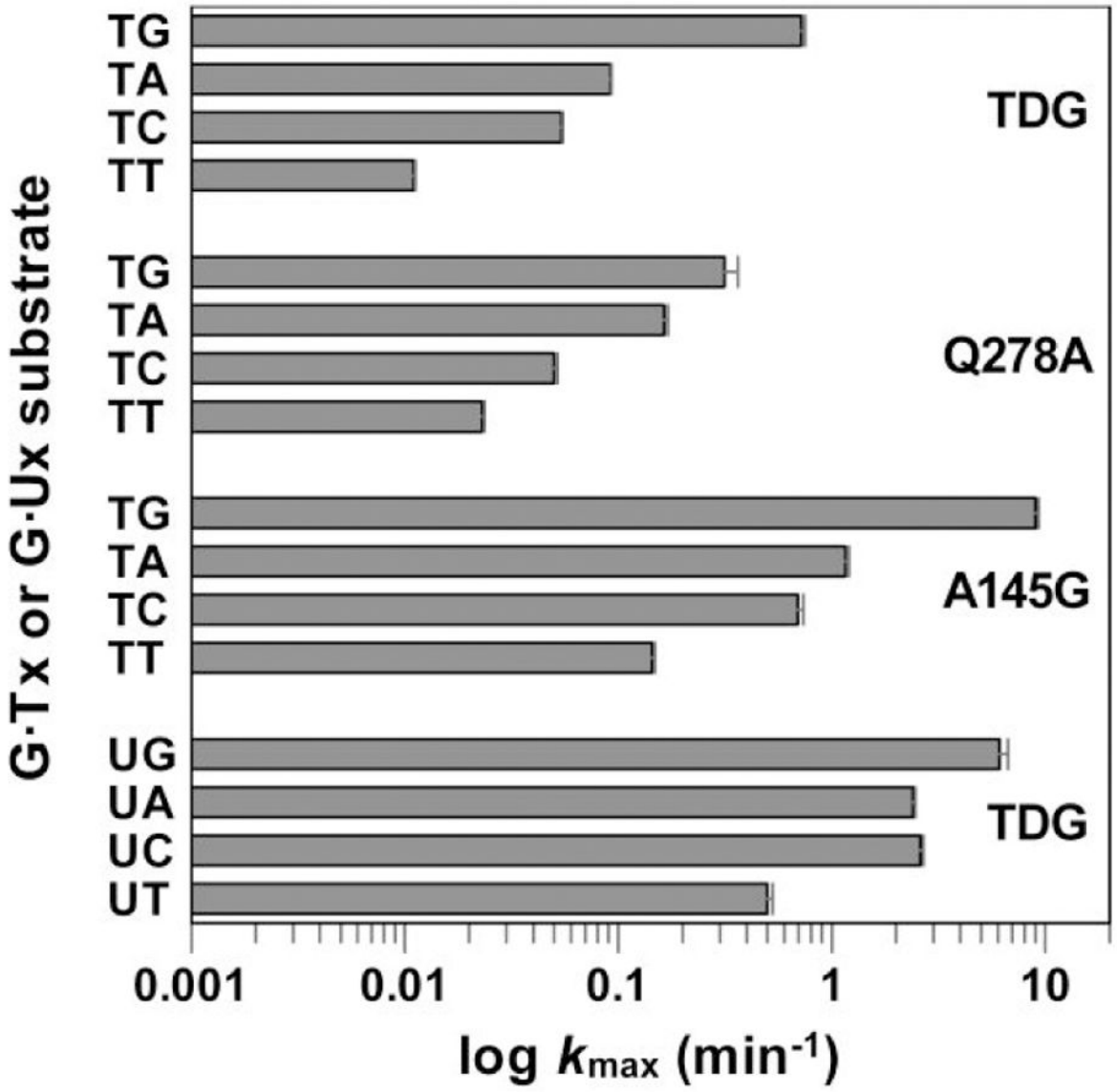


Figure 5.

Thymine excision activity (log k_{max}) of TDG and two variants acting on G·Tx or G·Ux substrates (abbreviated as Tx, Ux) at 37 °C. For all substrates the base at +1 varied and the base at +2 was T (G·TxT, G·UxT). Data fitting and k_{max} values are given in Supporting Information Figure S2, Table S2.

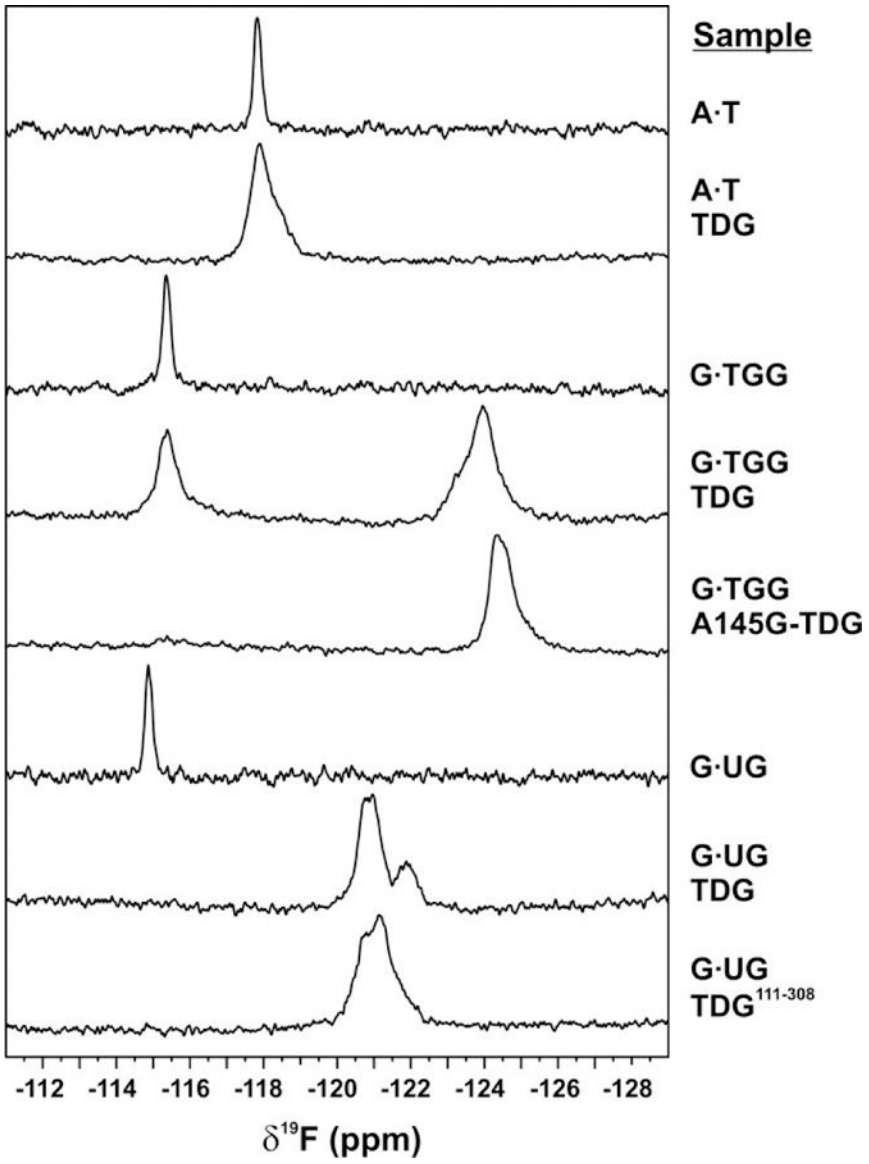


Figure 6.

^{19}F NMR spectra for samples of DNA containing dT$^{2'F}$ or dU$^{2'F}$, in the base pairs as indicated, with or without TDG, collected at 18 °C. Labels (right) indicate the type of DNA and enzyme (if present) in the NMR sample. One sample contained TDG$^{111-308}$ while others contained the TDG$^{82-308}$ construct (TDG, A145G-TDG).

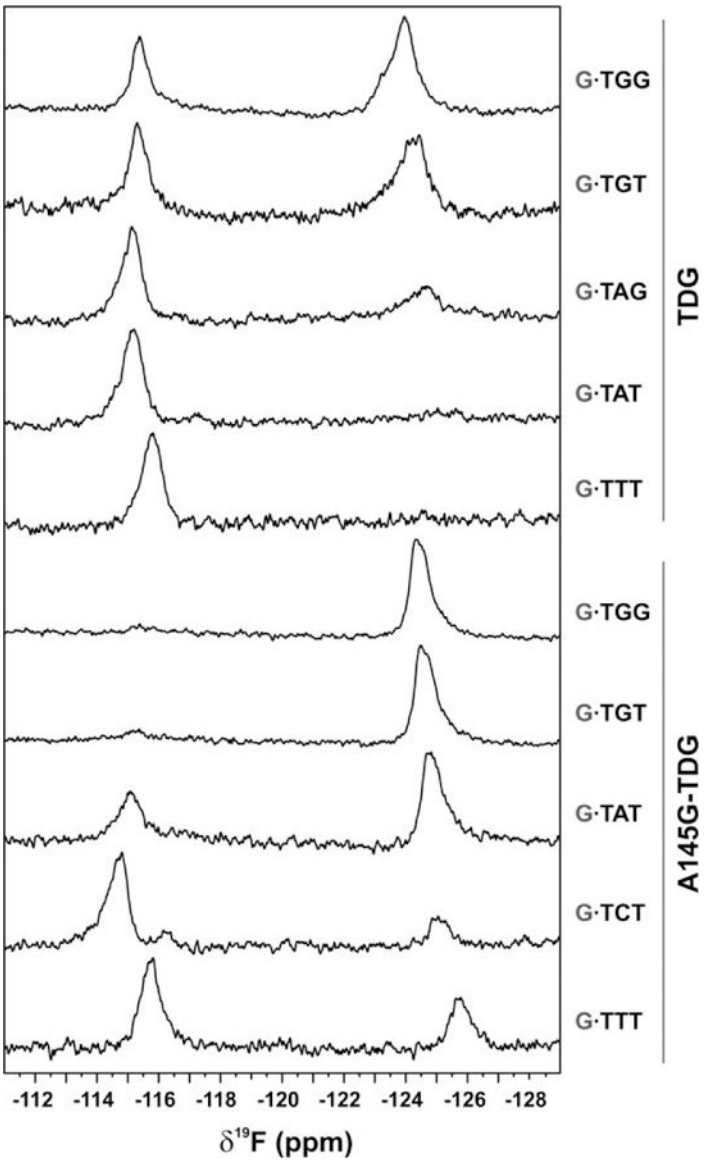


Figure 7.

^{19}F NMR spectra for 2′-F-dT in G·Txy DNA, bound to either TDG (top half) or to A145G-TDG (bottom half), collected at 18°C. Downfield peaks (near −115 ppm) reflect the stacked (non-flipped) conformation of 2′-F-dT, while the upfield peaks (near −125 ppm) report on the flipped conformation.

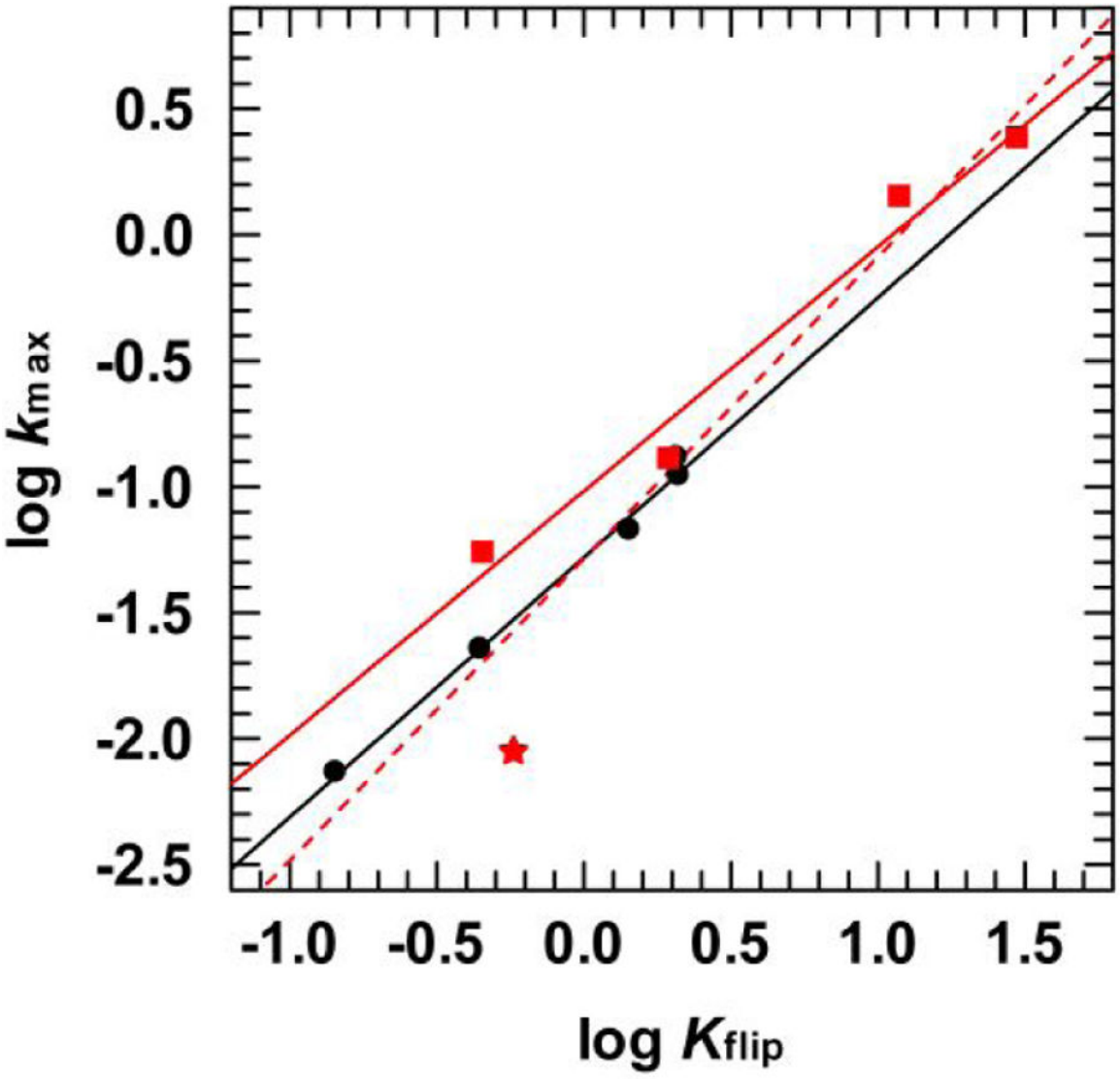


Figure 8.

Dependence of thymine excision activity (log k_{max}) on nucleotide flipping (log K_{flip}) for TDG or A145G and G·Txy substrates, at 18 °C. Linear fitting gives a slope of $m = 1.03 \pm 0.05$ ($r = 0.996$) for TDG (●, black line). Data for A145G-TDG (■) were fitted with or without G·TTT (★). Fitting that excludes G·TTT (solid red line) gives a slope of $m = 0.97 \pm 0.11$ ($r = 0.988$); fitting that includes G·TTT (dotted red line) gives a slope of $m = 1.20 \pm 0.23$ ($r = 0.949$). Data fitting and k_{max} and K_{flip} values are in Supporting Information Table S3, Figure S3.

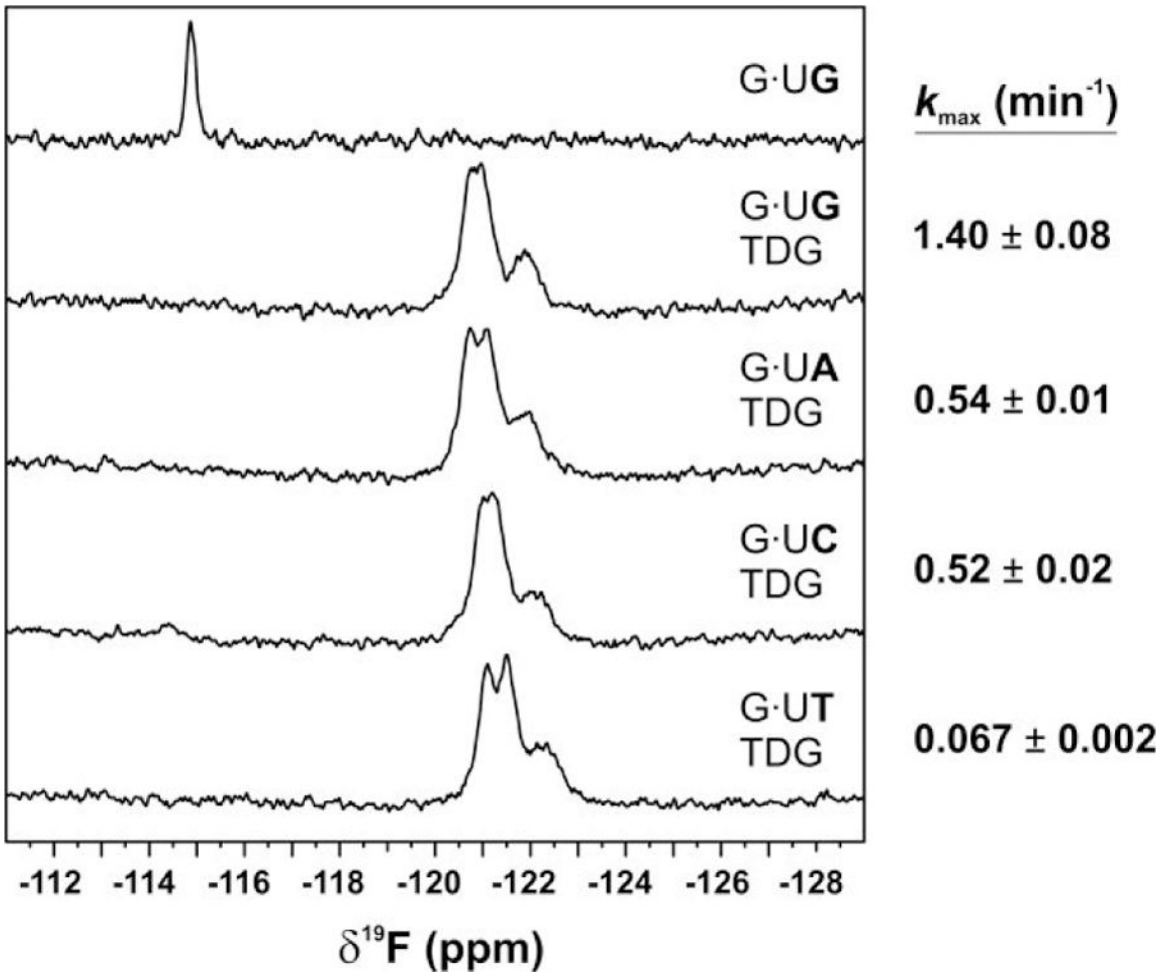


Figure 9.

Analysis of dU nucleotide flipping and uracil excision at 18 °C. ^{19}F NMR spectra for dU$^{2'F}$ in G·Ux DNA, free and TDG-bound. Uracil excision activity (k_{max}) for G·Ux substrates (at 18 °C) is shown to the right of corresponding NMR spectra.

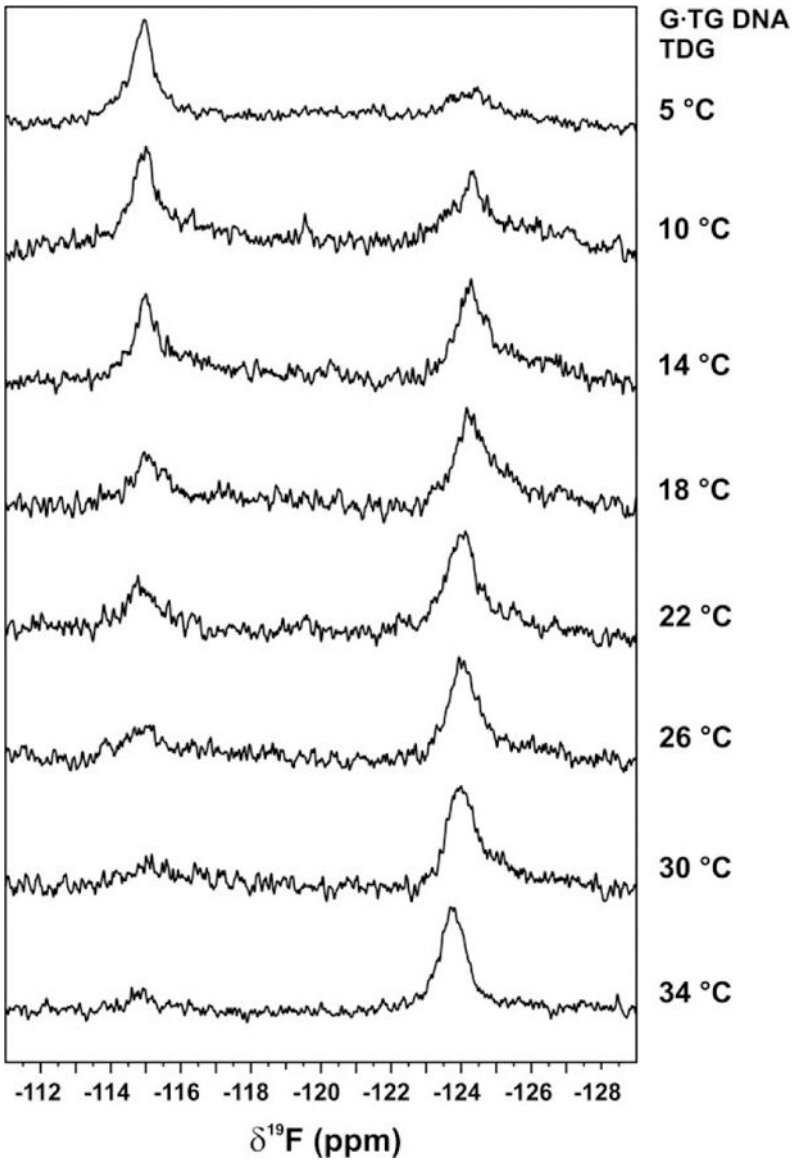
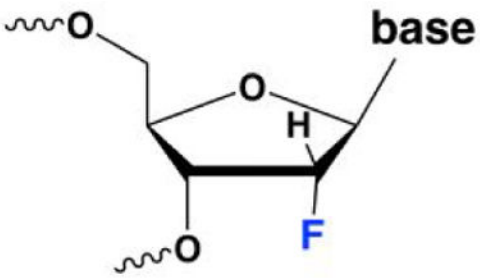
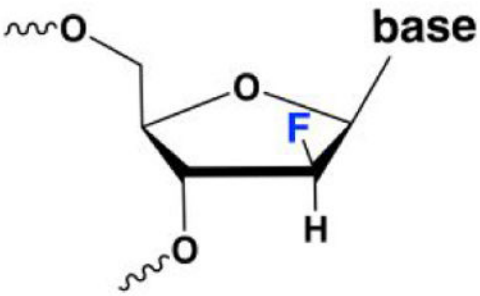


Figure 10.

^{19}F NMR spectra for dT$^{2'F}$ in G·TGC DNA (0.15 mM) bound to TDG (0.3 mM) collected with varying temperature. The spectra exhibit two peaks, reporting on the stacked state (downfield) and the flipped state (upfield) for dT$^{2'F}$.



2′-F-ribo (α)



2′-F-araribno (β)

Chart 1.

Two types of 2′-F substitutions in deoxynucleotides. Studies here used the 2′-F-arabino (2′-F-β) forms of dT or dU, referred to as $dT^{2'F}$ and $dU^{2'F}$.