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Dependence of substrate binding and catalysis on pH, ionic strength, and temperature for thymine DNA glycosylase: Insights into recognition and processing of G-T mispairs

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Abstract

Repair of G-T mismatches arising from deamination of 5-methylcytosine (m^5C) involves excision of thymine and restoration of a G-C pair via base excision repair (BER). Thymine DNA glycosylase (TDG) is one of two mammalian enzymes that can specifically remove thymine from G-T mispairs. While TDG can excise other bases, it maintains stringent specificity for a CpG context, suggesting deaminated m^5C is an important biological substrate. Recent studies reveal TDG is essential for embryogenesis; it helps maintain an active chromatin complex and initiates BER to counter aberrant *de novo* CpG methylation, which may involve excision of actively deaminated m^5C . The relatively weak G-T activity of TDG has been implicated in the hypermutability of CpG sites, which largely involves C→T transitions arising from m^5C deamination. Thus, it is important to understand how TDG recognizes and process substrates, particularly G-T mispairs. Here, we extend our detailed studies of TDG by examining the dependence of substrate binding and catalysis on pH, ionic strength, and temperature. Catalytic activity is relatively constant for pH 5.5-9, but falls sharply for pH >9 due to severely weakened substrate binding, and, potentially, ionization of the target base. Substrate binding and catalysis diminish sharply with increasing ionic strength, particularly for G-T substrates, due partly to effects on nucleotide flipping. TDG aggregates rapidly and irreversibly at 37 °C, but can be stabilized by specific and nonspecific DNA. The temperature dependence of catalysis reveals large and unexpected differences for G-U and G-T substrates, where G-T activity exhibits much steeper temperature dependence. The results suggest that reversible nucleotide flipping is much more rapid for G-T substrates, consistent with our previous findings that steric effects limit the active-site lifetime of thymine, which may account for the relatively weak G-T activity. Our findings provide important insight into catalysis by TDG, particularly for mutagenic G-T mispairs.

Keywords

base excision repair; 5-methylcytosine deamination; G-T mispair; CpG sites; fluorescence

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Conflict of Interest

The authors declare there are no conflicts of interest.

1. Introduction

While the repair of G·T mismatches generated by replication errors is directed at the new strand, repair of those caused by deamination of 5-methylcytosine (m^5C) must involve the removal of T and restoration of a G·C base pair. Two mammalian enzymes exhibit specificity for excising T (but not G) from G·T mismatches, thymine DNA glycosylase (TDG) [1,2], the focus of this work, and methyl binding domain IV (MBD4) [3,4]. Downstream base excision repair proteins complete the process of restoring a G·C base pair. TDG can also remove a broad range of other bases from DNA *in vitro* (uracil, 5-halouracils, among others), often with greater efficiency than it exhibits for G·T mismatches [5,6]. However, without exception, mammalian TDG exhibits stringent specificity for removing bases that are paired with guanine rather than adenine and that are located in a CpG dinucleotide sequence context [7-10]. Of the many bases that can be efficiently removed by TDG, only deaminated m^5C is likely to arise selectively in a CpG context. These findings suggest G·T mismatches arising by m^5C deamination are an important biological substrate for TDG [9]. This conclusion is supported by studies of MBD4 deficient mice, which reveal an increase in C→T transitions at CpG sites but also indicate that factors other than MBD4 contribute to the repair of deaminated m^5C [11,12]. As mentioned above, TDG is the only other mammalian enzyme known to have specificity for initiating repair of G·T mismatches created by m^5C deamination.

Many studies indicate a role for TDG in regulating gene expression, and this appears to involve both its catalytic activity and interactions with proteins involved in transcriptional regulation. Cytosine methylation (m^5C) is an epigenetic signal for gene silencing, and maintaining the appropriate methylation status of CpG dinucleotides and chromatin structure is essential for proper regulation of gene expression and for embryonic development [13]. While it is established that cytosine 5-methyltransferases convert C to m^5C , the mechanism for reversing this modification has remained unclear. Previous studies suggest a role for TDG and BER in the active demethylation of CpG sites, which may involve excision of deaminated m^5C by TDG [14-18]. A recent study reveals TDG is essential for embryonic development and indicates it is required for maintaining epigenetic stability of genes expressed in development, by contributing to the assembly of chromatin modifying complexes and by initiating BER to counter aberrant *de novo* CpG methylation [19]. The requirement for TDG catalytic activity seems likely to involve excision of *deaminated* m^5C , because TDG has exceedingly weak activity for removing m^5C [19]. Demethylation could potentially involve conversion of m^5C to 5-hydroxymethylcytosine (hmC) by TET proteins [20]. Our previous work suggests TDG would also have weak activity for excising hmC, though it has substantial activity for excising the deamination product of hmC, 5-hydroxymethyluracil, particularly from a CpG context [6].

Together, these findings indicate the importance of obtaining a detailed understanding of how TDG recognizes and processes substrates, particularly G·T mismatches. Deamination of m^5C is considered a major source of C→T transitions at CpG sites, which are among the most frequent point mutations in cancer and genetic disease [21-24]. While the findings above suggest G·T mismatches arising from m^5C deamination are an important biological substrate of TDG, many studies have shown that binding and catalysis is substantially weaker for G·T mismatches relative to other TDG substrates that have been identified by *in vitro* studies [8,10,25,26]. It is important to understand why this is the case, to further our knowledge of the TDG catalytic mechanism, and because it may be relevant to the high mutational frequency observed at CpG sites [27].

Unlike the vast majority of DNA glycosylases, TDG and MBD4 face the formidable challenge of removing a normal base from a mismatched base pair. The relatively weak G·T

activity exhibited by these enzymes might reflect a compromise between the need for efficient processing of deaminated m⁵C and for avoiding aberrant removal of T from A·T pairs [11,27]. Consistent with this hypothesis, our previous findings suggest the TDG active site offers limited access to thymine and other bases with bulky C-5 substituents, due likely to steric hindrance [6,10,26,28]. Indeed, our findings indicate that the much lower catalytic activity observed for G·T relative to G·U substrates can be largely explained by steric hindrance involving the methyl group of thymine, although stability of the scissile *N*-glycosylic bond is likely a contributing factor, as the *non-enzymatic* hydrolysis is ~3-fold faster for dU relative to dT (at 22 °C) [6,29]. Likewise, steric effects seem likely to account largely for the 30-fold weaker binding affinity of TDG for G·T relative to G·U substrates [28]. Moreover, the much lower catalytic activity for the excision of 5-BrU versus 5-FU can be attributed largely to steric effects involving the C-5 substituent, because the *N*-glycosylic bond is of nearly equivalent stability for BrdU and FdU [6,10].

Here, we extend our detailed studies of how TDG recognizes and processes its substrates, particularly G·T and G·U mispairs, and further explore the molecular basis of its relatively weak G·T activity. We rigorously examined the effects of varying pH, ionic strength, and temperature on binding and catalysis for G·T and G·U substrates, using single turnover kinetics and equilibrium binding experiments (monitored by fluorescence anisotropy). Such studies have not previously been reported for any member of the large TDG-MUG family of enzymes. The pH dependence of substrate binding and catalysis can provide important insight into enzyme mechanism [30], as shown for uracil DNA glycosylase (UNG) and alkyladenine DNA glycosylase (AAG) [31,32]. We find dramatic and unexpected differences in the temperature dependence of catalysis for G·T and G·U substrates, which are consistent with the hypothesis that G·T activity is limited by steric effects involving the methyl group of thymine. While many previous studies of TDG have been performed at physiological temperature, the stability of TDG at 37 °C had not been established. Accordingly, we examined the thermal stability of TDG using CD and fluorescence spectroscopy, and in the presence and absence of specific and nonspecific DNA. The findings have important implications for obtaining experimental conditions that assure enzyme activity, and conditions that provide maximal activity, including considerations of temperature, ionic strength, and the presence of DNA. Together, our findings provide important new insight into the catalytic mechanism of TDG, including recognition and processing of G·T mispairs arising from m⁵C deamination.

2. Materials and Methods

2.1. DNA synthesis and purification

The 16 bp DNA construct, used for most experiments, contained 5'-CTCAxGTACAGAGCTG, where x is the target nucleotide (x = T, T^F, U, U^F), and 5'-CAGCTCTGTACGTGAG, such that the target nucleotide is paired with G and located in CpG context (Supplementary Fig. 1). Similarly, the 28 bp DNA contained 5'-GTGTCACCACTGCTCAxGTACAGAGCTG and 5'-CAGCTCTGTACGTGAGCAGTGGTGACAC. The 32 bp G·T substrate has the same construct as the 28 bp DNAs, with GC nucleotides added at each terminus. The 28 bp nonspecific DNA (NS28) is nearly identical but does not contain a mismatch or a CpG site. Oligonucleotides were synthesized and purified as described [26], with purity verified by ion-exchange HPLC [6]. Duplex DNA was hybridized by rapid heating to 80 °C followed by slow cooling to room temperature. DNA used for fluorescence anisotropy was labeled with sulforhodamine (Texas Red, TR) in the non-target strand (5' amino C6 modifier) [28], which was synthesized and purified (RP-HPLC) by Midland Certified Reagent Co. (Midland, TX). Oligonucleotides containing the dT analog 2'-deoxy-2'-flouroarabinothymidine (T^F, Supplementary Fig. 1) and the dU analog 2'-deoxy-2'-flouroarabinouridine (U^F) were

obtained as described [26]. Control experiments demonstrate DNA containing T^F or U^F is completely resistant to cleavage by TDG [26], consistent with previous findings [33,34]. These analogs differ from the natural dT and dU nucleotides only by replacement of 2'-H with fluorine (2'-fluoroarabino), which renders the *N*-glycosylic bond of these and other nucleotides highly resistant to enzymatic cleavage [26,31,33,34]. Previous studies show the 2'-fluoroarabino substitution, in dT and other deoxynucleotides, promotes an O4'-endo sugar pucker, which is fully compatible with B-DNA geometry [35-37]. Although the O4'-endo conformation of T^F could potentially alter TDG binding (relative to binding for natural dT), our previous studies indicate any such effect is small [26].

2.2 Enzyme Purification

Human TDG was expressed and purified as previously described [10,38], quantified by absorbance using $\epsilon^{280} = 31.5 \text{ mM}^{-1}\text{cm}^{-1}$, flash frozen, and stored at -80°C . TDG(56-308) was purified essentially as previously described for TDG(111-308) [38], quantified by absorbance ($\epsilon^{280} = 17.4 \text{ mM}^{-1}\text{cm}^{-1}$), flash frozen, and stored at -80°C .

2.3 Kinetics experiments

Kinetics experiments were collected under single turnover conditions ($[\text{TDG}] \gg [\text{substrate}]$) in order to eliminate effects of product release or product inhibition on the observed rate constants (k_{obs}). This is important because TDG exhibits very slow product release and strong product inhibition, which dominates k_{cat} values determined under steady-state (multiple turnover) conditions [8,25,39]. Experiments were collected in HEMN.1 buffer (0.01 M HEPES, 0.2 mM EDTA, 2.5 mM MgCl₂, 0.1 M NaCl) at 22°C , unless otherwise noted. Experiments were initiated by adding concentrated TDG to buffered substrate, followed by rapid mixing. At various time points, aliquots were removed, quenched with 50% (v:v) 0.3M NaOH and 0.03M EDTA, and heated at 85°C for 15 min to cleave the DNA backbone at enzyme-produced abasic sites. The fraction product was determined by HPLC [6,10], and data were fitted to eq. 1 using non-linear regression with Grafit 5:[40]

$$\text{fraction product} = A(1 - e^{-kt}) \quad (1)$$

where A is the amplitude, k is the observed rate constant, and t is the reaction time (min).

2.4 Equilibrium binding monitored by fluorescence anisotropy

We used fluorescence anisotropy to determine equilibrium dissociation constants for TDG binding to G·T^F and G·U^F substrate analogs (10 nM and 0.5 nM, respectively) as described [28], monitoring the fluorescence of sulforhodamine (Texas Red, or TR) at the 5'-end of the non-target DNA strand, using a QuantaMaster 40 spectrofluorometer (PTI, Birmingham, NJ). Equilibrium dissociation constants for TDG binding to the G·T^F substrate analog were determined by fitting anisotropy data from at least two independent binding experiments using DynaFit 4 [41,42] as previously described [28] and detailed below. Our previous studies show TDG binds DNA containing a G·T substrate analog initially with 1:1 stoichiometry and a second TDG subunit can bind for high and excess TDG concentrations, giving a 2:1 complex [28]. The same behavior is observed for TDG binding to DNA containing other specific sites (G·U mispair, G·AP product, undamaged CpG site) [28]. As we recently described, a benefit of using DynaFit for fitting data to complex binding models is that fitting does not require an analytical equation. Accordingly, binding experiments are not subject to restraints imposed by assumptions of an analytical equation (i.e., the concentration of labeled DNA is far below K_d). Thus, binding experiments can be collected

using a concentration of labeled DNA that approximates the lowest K_d value. A representative example of the scripts used for data fitting, including the 1:1 and 2:1 binding models, is provided in Supplementary Data. The fitted parameters include the dissociation constants (K_{d1} , K_{d2}) and anisotropy values for free DNA (r_D) and enzyme-DNA complexes with stoichiometry of 1:1 (r_{ED}) and 2:1 (r_{EED}). We fitted the data using a fixed value of r_{ED} (0.20), because if r_{ED} is not fixed, K_{d2} and r_{ED} are poorly constrained and unreasonably high. The choice of $r_{ED} = 0.20$ is based on the r_{ED} value obtained from fitting anisotropy data for TDG binding to similar DNA containing a G·U or a G·AP site (for which all parameters are well constrained) [28]. In all cases, a superior fit was obtained for the 2:1 rather than 1:1 model, based on visual inspection and/or the probability value of the Fisher's F-statistic (obtained from fitting), where $p < 0.05$ is considered significant.

2.5 Fluorescence spectroscopy to monitor conformational changes in TDG

Fluorescence emission spectra of TDG were collected in various buffers (below), to monitor the effects of temperature, pH, and ionic strength on the structure of TDG. An excitation wavelength of 295 nm was used to excite Trp and minimize excitation of Tyr [43], because the 15 Tyr residues of TDG contribute strongly to fluorescence for excitation at 280 nm. Emission spectra for TDG (1 μ M) were typically collected over the range of 310 nm to 410 nm, at 1 nm intervals with integration time of 1 sec. Spectra collected to measure effects of pH or ionic strength are the average of two scans. To allow rapid data acquisition for experiments at 37 °C, spectra were recorded from 315 nm to 365 nm with a 0.2 s integration time.

2.6 Circular dichroism (CD) spectroscopy

Thermal melting experiments for TDG (2.5 μ M) were conducted with a Jasco J-715 spectropolarimeter (Easton, MD) with a Peltier for temperature control and a 1 cm path length cuvette, monitoring ellipticity at 222 nm (CD_{222}). Data were collected from 10 °C to 60 °C, with a temperature gradient of 1 °C per min. The buffer was 0.01 M Tris-HCl pH 7.5, 0.1 M NaCl, 0.5 mM DTT. In addition to CD_{222} , the spectropolarimeter simultaneously records the high voltage applied to dynodes of the photomultiplier (PM voltage). The PM voltage increases with a reduction in light intensity, due to absorption and/or scattering. Because ϵ^{222} exhibits small temperature dependence, no significant change in absorption is expected, and changes in PM voltage are attributed to light scattering [44,45].

2.7 Buffers for studying the effects of pH and ionic strength on TDG structure and activity

The effect of pH on G·T substrate binding, catalytic activity, and TDG structure (Trp fluorescence) was determined using a 0.01 M concentration of an appropriate buffer (see below) supplemented with 0.1 M NaCl, 2.5 mM $MgCl_2$, and 0.2 mM EDTA. The buffers used for various pH values were [31]: sodium phosphate, pH 2; sodium acetate, pH 4-5.5; NaMES, pH 5.5-6.8; NaHEPES, pH 6.8-8.0; Tris-HCl, pH 8.0-9.0; NaCHES, pH 9.0-10.0; NaCAPS, pH 10.0-11.0; sodium phosphate, pH 12 (without $MgCl_2$ due to formation of insoluble magnesium hydroxide). Buffers for studying the effect of increasing ionic strength on binding and catalysis contained 0.01 M HEPES (pH 7.5), 2.5 mM $MgCl_2$, 0.2 mM EDTA, and sufficient NaCl to give the desired ionic strength.

3. Results

3.1 Experimental Approach

Previous kinetics studies of TDG and MUG enzymes show that product release is very slow and product inhibition is potent, such that k_{cat} values obtained from steady-state kinetics are dominated by steps that occur *after* the chemical step (k_{chem}) [25,39,46]. Accordingly, we

used single turnover kinetics experiments, which yield rate constants (k_{obs}) that report on nucleotide flipping (K_{flip}) and k_{chem} , but are *not* influenced by steps after chemistry [6,25]. Nucleotide flipping is a conformational change used by glycosylases whereby the target nucleotide flips out of the DNA and into the enzyme active site to give the reactive (Michaelis) complex. We used 16 bp G·U and G·T substrates, with the mispair in a CpG sequence context, consistent with TDG specificity (Supplementary Fig. 1). The 16 bp substrates used here satisfy all TDG-DNA interactions observed in a crystal structure of TDG (catalytic domain) bound to DNA, and they provide full catalytic activity under saturating enzyme conditions, consistent with our previous results demonstrating that 2:1 binding does not contribute to catalytic activity for G·T or G·U substrates [28,38]. However, because the binding affinity is somewhat tighter for 28 bp relative to 16 bp G·T substrates [28], due likely to nonspecific interactions involving the disordered N-terminal region of TDG [47], we used a longer G·T substrate for some experiments, which provided essentially the same result as the 16 bp G·T substrate.

To study the effects of pH and ionic strength on TDG binding to substrates in the absence of base cleavage, we used G·T^F and G·U^F substrate analogs, where T^F and U^F are close mimics of dT and dU that bind specifically but are not cleaved by TDG (Supplementary Fig. 1) [26,33]. The equilibrium binding experiments, monitored by fluorescence anisotropy, provide a dissociation constant (K_{d1}) that reports on at least two steps, formation of the initial TDG-DNA complex (K_{ES}) and nucleotide flipping (K_{flip}) [28]. As we have shown previously, TDG can form a 2:1 complex with DNA under conditions of high and excess concentrations of TDG, where one TDG subunit binds the specific site (i.e., G·T^F) with high affinity (K_{d1}) and a second TDG subunit binds nonspecifically with much weaker affinity (K_{d2}) [28,38]. Our studies also showed that under limiting enzyme (multiple turnover) conditions, TDG binds and processes G·T substrates using 1:1 stoichiometry. Accordingly, our interest here is the effect of pH and ionic strength on the affinity of TDG for the specific site (K_{d1}) rather than the nonspecific site (K_{d2}), although both are reported.

3.2 pH dependence of catalysis

We determined the pH dependence of catalysis for TDG acting upon G·T and G·U substrates using single turnover experiments, as shown in Fig. 1A. TDG activity for the G·U substrate is relatively constant for pH 5.5-9.0, falls 90-fold for pH 9.0-10, and no activity is detected at pH 10.5. At pH 5.0, G·U activity is observed initially (up to 20 s) but it dissipates before the reaction is complete, suggesting an inactivating TDG conformational change occurs (within 20 s) at pH 5.0, consistent with results shown below. This is not due to an effect of the buffer (sodium acetate), because full activity is observed in the same buffer at pH 5.5.

For the G·T substrate and a fixed (2.5 μM) concentration of TDG, catalytic activity is fairly constant for pH 6.5-7.5, falls 6-fold from pH 7.5-9, and activity is not detected at pH 9.5. For pH 5.5, activity is observed initially (up to 2 min) but dissipates before the reaction is complete, indicating a slow loss of TDG activity at pH 5.5. (The G·U reaction is complete in <1 min at pH 5.5). No G·T activity is observed at pH 5.0, consistent with results for G·U. Similar results are obtained for a longer G·T substrate (Fig. 1A). Notably, the loss of G·T activity at pH 9 can be largely recovered by using a higher concentration of TDG (Fig. 1A), consistent with findings below that substrate binding is severely weakened with increasing pH. When the data collected at pH 9 with a higher TDG concentration are considered, the pH profiles for G·T and G·U activity are quite similar over the range of pH 6-9 (Fig. 1A).

3.3 pH dependence of substrate binding

To examine the role of substrate binding in the loss of G·T activity for pH >7.5, we performed equilibrium binding experiments, monitored by fluorescence anisotropy with a

labeled G·T^F substrate analog, as shown in Fig. 1B. At pH 7.5, TDG binds the G·T^F analog with a dissociation constant of $K_{d1} = 0.12 \pm 0.02 \mu\text{M}$. The binding affinity is nearly four-fold weaker at pH 8.5, $K_{d1} = 0.42 \pm 0.05 \mu\text{M}$, and 36-fold weaker at pH 9.5, $K_{d1} = 4.4 \pm 0.5 \mu\text{M}$. A very similar result is observed for a longer G·T substrate (Supplementary Fig. 2A). We also examined the effect of increasing pH on G·U substrate binding. At pH 7.5, TDG binds a G·U^F analog tightly, $K_{d1} = 0.008 \pm 0.003 \mu\text{M}$ [28], but the affinity is 30-fold weaker at pH 9.0, $K_{d1} = 0.244 \pm 0.046 \mu\text{M}$ (Supplementary Fig. 2B). While G·U substrate binding is much weaker at pH 9.0, the concentration of TDG used in the kinetics experiments above (2.5 μM) is sufficient to saturate the substrate such that activity is not diminished (Fig. 1A). In contrast, G·T substrate binding is sufficiently weakened to affect G·T catalytic activity for pH >7.5-9, consistent with the finding that G·T activity can be recovered at pH 9.0 by using a higher concentrations of TDG (Fig. 1A).

3.4 Effects of varying pH on TDG structure

When catalytic activity decreases at high or low pH, it is important to determine whether the loss may be due, at least in part, to structural perturbations of the enzyme. We used tryptophan fluorescence to monitor pH-induced conformational changes in TDG. The emission energy (λ_{max}) of Trp is highly sensitive to polarity; λ_{max} is about 355 nm for Trp in water, while λ_{max} can be as low as 310 nm for Trp buried in the hydrophobic interior of a protein [43,48]. Protein unfolding invariably leads to an increase in λ_{max} for a buried Trp, while the effect on quantum yield (intensity, I_{max}) is less predictable [43]. TDG contains two Trp residues, Trp160, buried in the catalytic domain [38,49], and Trp383, in the C-terminal region, which is intrinsically disordered [50]. As indicated in Supplementary Fig. 3, emission from Trp383 dominates the fluorescence of TDG. Thus, to focus on pH-induced conformational changes in the *catalytic domain*, we used TDG(56-308), which contains only Trp160. Importantly, TDG(56-308) exhibits essentially the same substrate binding and catalytic activity as full-length TDG [47,50-52]. As shown in Fig. 2, fluorescence spectra for TDG(56-308) at pH 7.5 reveal Trp160 exhibits $\lambda_{\text{max}} = 315 \text{ nm}$, indicating a rather hydrophobic environment. λ_{max} is relatively constant for pH 6-9, suggesting no substantial conformational change, consistent with the flat pH profile for pH 6-9. The small increase in λ_{max} and changes in I_{max} for pH 5.5, 9.5, and 10 suggest a modest conformational change (Supplementary Fig. 3D), consistent with diminished G·U activity and absence of G·T activity for pH > 9, and loss of activity within minutes at pH 5.5. Large increases in λ_{max} (and changes in I_{max}) for pH ≤ 5 and pH ≥ 10.5 indicate unfolding of TDG, consistent with the absence of catalytic activity at these pH extremes.

3.5 Dependence of substrate binding and catalysis on ionic strength

We also examined the effect of increasing ionic strength on TDG activity. As shown in Fig. 3A, activity for G·U substrates is relatively constant for ionic strength of 0.01 to 0.2 M, falls dramatically as ionic strength increases to 0.4 M, and no activity is detected at 0.6 M ionic strength. The effects of increasing ionic strength are even larger for G·T activity. Relative to the activity at 0.06 M ionic strength, activity is modestly lower at 0.11 M, 4-fold lower at 0.16 M, 10-fold lower at 0.2 M, 120-fold lower at 0.3 M, and activity is not detected at 0.4 M. The results are essentially the same for a 32 bp G·T substrate (Fig. 3A). The loss in G·T activity at 0.16 M ionic strength is *not* recovered by increasing the concentration of TDG by four-fold (Fig. 3A), indicating that increasing ionic strength impairs nucleotide flipping and/or the chemical step of the TDG reaction, in addition to effects on the initial association of TDG and substrate (as discussed below). Fluorescence studies indicate the diminished activity is not due to substantial conformational changes induced by increasing ionic strength (Supplementary Fig. 4), as expected. Notably, free TDG aggregates at very low ionic strength, 0.01 M (Supplementary Figs. 4 and 5), but observation that it retains full

catalytic activity at this ionic strength indicates that binding to DNA substrate (or product) suppresses aggregation at low ionic strength.

To assess the extent to which the loss in catalytic activity with increasing ionic strength is due to an effect on substrate binding, we examined the dependence of substrate binding on ionic strength, using fluorescence anisotropy. As shown in Fig. 3B, the affinity of TDG for a G-T^F substrate analog depends strongly on ionic strength. TDG exhibits high affinity for the G-T^F analog at 0.06 M ionic strength, $K_{d1} = 0.024 \pm 0.003 \mu\text{M}$, but the affinity is five-fold weaker at 0.11 M, $K_{d1} = 0.12 \pm 0.02 \mu\text{M}$, 46-fold weaker at 0.16 M (near physiological), $K_{d1} = 1.1 \pm 0.1 \mu\text{M}$, and exceedingly weak at 0.3 M. A very similar result is observed for a longer G-T substrate (Supplementary Fig. 4A). Together, the binding and kinetics results indicate the effect of increasing ionic strength on catalytic activity involves effects on nonspecific binding (K_{ES}) and nucleotide flipping (K_{flip}), as discussed below.

3.6 Temperature dependence of catalytic activity

We determined the temperature dependence of TDG catalytic activity, and find striking and unexpected differences between G-U and G-T substrates. As shown in Fig. 4, catalytic activity increases with temperature from 5 °C to 37 °C, with much steeper temperature dependence for G-T relative to G-U substrates. Indeed, G-T activity increases 11-fold from 15 °C to 37 °C, compared to a 3-fold increase for G-U activity. Notably, G-T activity is five-fold higher at 37 °C relative to 22 °C, the two temperatures used most frequently in studies of TDG. These findings suggest important differences in recognition and processing of G-T relative to G-U substrates, as discussed below. Remarkably, two phases are observed in the temperature dependence for G-T activity; the phase discussed above (from 15-37 °C) and an even steeper phase from 5-15 °C. Identical results are obtained at 5 °C and 10 °C when the experiments are repeated with four-fold higher concentrations of enzyme and substrate, indicating saturating enzyme conditions. In addition, the same temperature dependence is observed for a longer G-T substrate. Thus, the sharp decrease in G-T activity observed for temperatures below 15 °C is due to an effect on nucleotide flipping and/or the chemical step, rather than association of enzyme and substrate. The potential mechanistic implications of these remarkable findings are discussed below.

3.7 TDG is unstable at 37°C in the absence of DNA

Knowledge of the thermal stability of a protein is clearly important for experimental design, but such studies had not been reported for TDG. While many previous kinetics and binding studies were conducted at 37 °C, the stability of TDG at 37 °C had not been clearly established. To examine the thermal stability of TDG, we performed a melting experiment using circular dichroism (CD) spectroscopy, monitoring ellipticity at 222 nm (CD_{222}). As shown in Fig. 5A, the CD melting curve indicates TDG is stable up to about 25 °C, begins to unfold above 25 °C, and largely unfolds from 30 °C to 45 °C. As discussed below, folding is irreversible, precluding a thermodynamic analysis (including a meaningful T_m determination).

To assess whether unfolding leads to aggregation, we simultaneously monitored CD_{222} and the photomultiplier (PM) voltage (Fig. 5B); the latter responds to changes in light absorption and/or scattering [45]. For proteins that undergo reversible unfolding, no substantial change in PM voltage is expected, because the extinction coefficient is not significantly temperature dependent and absorption therefore remains relatively constant. In contrast, if unfolding leads rapidly to aggregation (turbidity), an increase in PM voltage is expected due to light scattering [45]. As shown in Fig. 5B, turbidity increases simultaneously with CD_{222} from 30 °C to 35 °C, indicating unfolding coupled to aggregation. The drop in PM voltage above 36 °C is attributed to precipitation of aggregated protein. Dynamic light scattering experiments

(Supplementary Fig. 6) confirm that TDG aggregates at 37 °C, and show that aggregation is irreversible. In contrast, light scattering experiments show TDG is stable for at least 5 hr at 22 °C.

We also examined the stability of TDG at 37 °C using Trp fluorescence of TDG, as shown in Fig. 5C. Rapid emission scans were collected following dilution of TDG into buffer at 37 °C. The spectrum collected immediately after dilution (15 sec) reflects the heat-induced conformational change, with little aggregation. Compared to the spectra at 22 °C, a small increase in λ_{max} is observed, suggesting the conformational change does not substantially increase solvent exposure of Trp160 or Trp383. At longer time points, λ_{max} remains constant and I_{max} increases, which is attributed to light scattering caused by aggregation, consistent with the results from CD and dynamic light scattering experiments. The change in I_{max} is largely complete in 3 min, and fitting the time dependence of I_{max} to an exponential equation indicates aggregation occurs with a rate constant of about 1 min^{-1} at 37 °C (for a 1 μM concentration of TDG).

Observation of robust TDG catalytic activity at 37 °C (Fig. 4) indicates that substrate binding stabilizes TDG against heat-induced unfolding. We used Trp fluorescence to examine the effect of DNA on the stability of TDG at 37 °C. As shown in Fig. 5D, a saturating concentration of G·T^F substrate analog stabilizes TDG for over 2 h at 37 °C, consistent with the tight binding of TDG to this DNA. TDG is also stabilized at 37 °C by DNA containing a G·U mismatch or an abasic site (not shown), consistent with even tighter binding to these lesions relative to G·T mismatches [26,28]. In addition, a saturating amount of nonspecific DNA stabilizes TDG for over 2 h at 37 °C (not shown), consistent with our previous findings of remarkably tight binding to nonspecific DNA ($K_d = 0.3 \mu\text{M}$) [28].

4. Discussion

4.1 pH dependence of catalysis and implications for the mechanism of TDG

Determining the pH dependence of catalysis can be important for understanding enzymatic reactions, but such studies had not been reported for a TDG-MUG family member. For the G·U substrate, TDG exhibits relatively constant activity for pH 5.5-9, sharply decreasing activity for pH 9-10, and no activity at pH 5 or 10.5. The absence of G·U activity at pH 5 and 10.5 can be explained by inactivating structural changes for TDG at these pH values (Fig. 2). The sharp drop in activity for pH 9-10 is likely due in part to severely diminished substrate binding at high pH (Fig. 3B, Supplementary Fig. 2). Contributing factors could also include deprotonation of uracil N3 ($\text{p}K_a = \sim 9.5$) [6,53] because the dU anion is expected to be a poor substrate [54,55], and/or structural perturbation of TDG, which might be indicated by modest changes in Trp fluorescence for pH 9-10 (Fig. 2). The pH profile obtained for the 16 bp substrate and a fixed (2.5 μM) TDG concentration suggests greater sensitivity to increasing pH for G·T relative to G·U substrates. However, the loss of G·T activity at pH 9 is almost fully recovered by using a higher TDG concentration, such that the pH profiles are roughly similar for G·T and G·U substrates for pH 6-9. The absence of G·T activity for pH > 9 likely reflects very large effects of increasing pH on substrate binding. Ionization of the target thymine ($\text{p}K_a^{\text{N}3} = \sim 9.9$) [6,53] might be a contributing factor.

Our results provide no evidence for the requirement of a *protonated* side chain in catalysis (i.e., a general acid), unless it has a $\text{p}K_a > 9$, nor evidence for an essential *unprotonated* group, unless it has a $\text{p}K_a < 5$. This result supports previous studies suggesting that a strictly conserved Asn is the only residue likely to be essential for catalysis in the TDG-MUG family of enzymes (N140 for human TDG) [26,34,38,49,56]. Notably, UNG enzymes have an essential Asp in the corresponding position, which serves to activate a water molecule for nucleophilic attack and/or stabilize the chemical transition-state of the reaction [54,55]. For

E. coli UNG, this Asp has a $pK_a = 6.2$, and catalytic activity is 10-fold *lower* at pH 5.5 relative to pH 7.5 [31]. Our finding that TDG retains full catalytic activity at pH 5.5 underscores the key differences in catalytic mechanism between members of the UNG enzyme superfamily.

4.2 Effects of increasing ionic strength on TDG activity

The catalytic activity of TDG (k_{obs}) decreases sharply with increasing ionic strength, particularly for G·T substrates, and this can be attributed in part to deleterious effects on nucleotide flipping (K_{flip}) or the chemical step (k_{chem}) of the TDG reaction [6,10,26]. We find a 4-fold loss in k_{obs} for G·T activity as ionic strength increases from 0.06 M to 0.16 M. Observation that activity is not recovered by using a higher enzyme concentration at 0.16 M ionic strength indicates saturating enzyme conditions, such that the decrease in k_{obs} is not due to an effect on enzyme-substrate association (i.e., formation of the initial E·S complex). Much larger decreases in k_{obs} are observed at higher ionic strength, although this is likely due in part to effects on enzyme-substrate association. Although increasing ionic strength can be expected to weaken nonspecific protein-DNA interactions, adverse effects on K_{flip} or k_{chem} for a DNA glycosylase reaction are not necessarily expected. Indeed, studies of AAG show relatively constant single-turnover activity as ionic strength increases up to 0.3 M [57]. In sharp contrast, TDG exhibits a 100-fold *decrease* in single turnover activity (G·T) at 0.3 M relative to 0.06 M ionic strength.

Deleterious effects of increasing ionic strength on nucleotide flipping could potentially involve disruption of electrostatic interactions formed with the flipped base in the active-site, or interactions involving Arg275, a side chain that is important for stabilizing nucleotide flipping, particularly for G·T substrates [26,38]. Increasing ionic strength could potentially slow the chemical step by perturbing electrostatic interactions that stabilize the chemical transition state. The greater effect of ionic strength on k_{obs} for G·T versus G·U suggests a larger adverse effect on K_{flip} for G·T processing, consistent with our previous findings that nucleotide flipping is inherently less stable for dT relative to dU nucleotides [26,28,38], or perhaps a larger adverse effect on k_{chem} for G·T versus G·U substrates.

In addition to the effects of increasing ionic strength on K_{flip} and/or k_{chem} indicated by the results above, the equilibrium binding studies reveal a large adverse effect on nonspecific DNA binding. This is indicated by the *45-fold* decrease in G·T binding affinity as ionic strength increases from 0.06 M to 0.16 M, and likely involves perturbation of contacts with DNA backbone phosphates, among other potential interactions. Notably, our findings reveal conditions of ionic strength (0.06 M) that allow for measurement of a wide range of substrate binding affinities, which could be important for structure-activity studies to determine the effect of enzyme mutations or substrate modifications on substrate binding affinity for TDG.

4.3 Effects of temperature on catalytic activity and enzyme stability

While many previous studies of TDG were performed at physiological temperature, to our knowledge, it had not been established whether TDG is stable at 37 °C. We find TDG aggregates rapidly and irreversibly at 37 °C, although it can be stabilized by a sufficient quantity of specific or nonspecific DNA. These results suggest caution when performing studies at 37 °C, to ensure TDG remains fully active for the duration of an experiment. Provided such conditions can be identified, the 5-fold higher activity of TDG at 37 °C versus 22 °C (for G·T substrates) may be experimentally useful, and yield results that are more physiologically relevant. Alternatively, we show that TDG is stable for several hours at 22 °C in the absence of stabilizing DNA.

TDG catalytic activity (k_{obs}) increases with temperature (5 °C to 37 °C) for G·T and G·U substrates, and the temperature dependence is much steeper for G·T activity. This key observation was unexpected, and suggests a dramatic difference in catalysis for these closely related substrates, which is considered below. We first note that for *non-enzymatic* *N*-glycosylic bond cleavage of dU and dT nucleosides, the thermodynamic parameters ($\Delta H^\ddagger$, $T\Delta S^\ddagger$) are highly similar [29,58]. The reactions are overwhelmingly enthalpic with a small entropic contribution ($\Delta H^\ddagger = 28.6$ kcal/mol, $T\Delta S^\ddagger = -1.9$ kcal/mol for dU, 25 °C), consistent with a highly dissociative mechanism [29,58,59]. Similarly, enzymatic *N*-glycosylic bond cleavage by UNG and MutY involves a stepwise mechanism [59-61]. This precedent and our previous studies suggest the TDG reaction is highly dissociative, perhaps stepwise [6,55], and activation parameters for the *chemical step* ($\Delta H^\ddagger$, $T\Delta S^\ddagger$) seem unlikely to differ dramatically for G·T and G·U substrates. Thermodynamic parameters for TDG catalysis (k_{obs}) can be obtained from an Arrhenius plot (Supplementary Fig. 7), which gives $\Delta H = 18.5 \pm 1.0$ kcal/mol, $T\Delta S = -2.5 \pm 1.0$ kcal/mol for G·T activity (for $T \geq 15$ °C), and $\Delta H = 8.5 \pm 0.6$ kcal/mol, $T\Delta S = -10.8 \pm 0.6$ kcal/mol for G·U activity. Because these parameters are derived from k_{obs} , they can include contributions from nucleotide flipping or an associated TDG conformational change (and are not shown as $\Delta H^\ddagger$, $T\Delta S^\ddagger$). One interpretation is that for G·T catalysis, nucleotide flipping is in rapid equilibrium and does not contribute substantially to k_{obs} , consistent with thermodynamic parameters that might be expected if k_{obs} is dominated by k_{chem} (i.e., small $T\Delta S^\ddagger$). For G·U catalysis, reverse nucleotide flipping could be much slower (due to favorable active-site interactions) and thereby contribute to k_{obs} , as suggested by a large entropic component for G·U catalysis. Our recent observations indicate reverse nucleotide flipping is indeed much faster than k_{chem} for G·T substrates and comparable to k_{chem} for G·U substrates (Fitzgerald ME and Drohat AC, manuscript in preparation). This could be due to steric effects with T (but not U) that shorten the lifetime of the flipped state. As discussed above (Introduction), our previous studies strongly suggest that steric effects weaken substrate binding and catalytic activity and for T and other bases with bulky substituents at the C-5 position (5-BrU, 5-iodoU, etc.) [6,10,26,28]. Steric effects with T might be needed to minimize excision of T from A·T pairs, and may account for the relatively weak G·T activity of TDG, which must balance the needs for efficient damage repair and avoidance of aberrant activity on undamaged DNA.

Another remarkable finding is the sharp transition in temperature dependence of catalysis (~15 °C) for G·T but not G·U substrates. Similar observations were reported for other enzymes, and in some cases the transition was correlated to an enzyme conformational change [62]. Previous studies also find that a transition is observed for some but not all substrates. It seems plausible that the transition could reflect a TDG conformational change that effects G·T but not G·U activity. The very steep temperature dependence of G·T catalysis for $T < 15$ °C may reflect a conformation of TDG in which steric effects strongly suppress G·T (but not G·U) activity, such that activity is limited by an enzyme conformational change that precedes catalysis. Of course, other factors could contribute, and additional structural and biochemical studies are needed to further explore these remarkable findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AP	apurinic/aprimidinic
BER	base excision repair
CD	circular dichroism
dU	2'-deoxyuridine
dT	2'-deoxythymidine
HPLC	high pressure liquid chromatography
TDG	thymine DNA glycosylase
m⁵C	5-methylcytosine
MBD4	methyl binding domain IV
MUG	mismatch-specific uracil DNA glycosylase
UNG	uracil DNA glycosylase

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Fig. 1.

pH dependence of catalysis and substrate binding. (A) Single turnover kinetics experiments were collected (at 22 °C) to determine the rate of base excision (k_{obs}) for TDG (2.5 μM) acting upon 16 bp G-U ($\circ$) or G-T (Δ) substrates (0.25 μM) as a function of pH. The rate was also determined at pH 9 (blue ∇) using 8-fold higher concentrations of TDG (20 μM) and 16 bp G-T substrate (2 μM). Data are also shown for a 32 bp G-T substrate (0.25 μM), collected with 2.5 μM TDG (red $\Diamond$). Buffers are given in Materials and Methods (ionic strength was 0.11 M). k_{obs} values represent the mean of at least three independent experiments. (B) Equilibrium binding of TDG to a G-T^F DNA substrate analog (10 nM) at 22 °C, and pH 7.5 ($\circ$), pH 8.5 ($\square$) or pH 9.5 (Δ), monitored by fluorescence anisotropy using sulforhodamine labeled G-T^F DNA. Data from two independent experiments were fitted to a two-site binding model, as described previously and in Materials and Methods [28], giving the following dissociation constants: pH 7.5 ($\circ$), $K_{\text{d1}} = 0.12 \pm 0.02 \mu\text{M}$, $K_{\text{d2}} = 0.98 \pm 0.17 \mu\text{M}$; pH 8.5 ($\square$), $K_{\text{d1}} = 0.42 \pm 0.05 \mu\text{M}$, $K_{\text{d2}} = 3.1 \pm 0.3 \mu\text{M}$; pH 9.5 (Δ), $K_{\text{d1}} = 4.4 \pm 0.5 \mu\text{M}$, $K_{\text{d2}} = 22 \pm 5 \mu\text{M}$. As seen here and discussed previously [28], the second TDG subunit binds with weak affinity for high and excess concentrations of TDG, and is probably not physiologically relevant.

Fig. 2.

Effect of varying pH on TDG structure monitored by Trp fluorescence. (A) Trp fluorescence spectra of TDG(56-308) (1 μ M) collected at 22°C in varying buffers, with pH indicated by color. TDG(56-308) contains a single Trp residue, Trp160, buried in the catalytic domain. (B) λ_{max} as a function of pH for TDG(56-308) ($\circ$) and TDG (Δ). Note that in 6M Gdn-HCl, λ_{max} = 350 nm for both TDG(56-308) and TDG (Supplementary Fig. 3).

Fig. 3.

Catalytic activity and substrate binding are sharply diminished by increasing ionic strength. (A) Single turnover kinetics experiments were collected (at 22 °C) to determine the base excision (k_{obs}) for TDG (5 μM) and G·U (○) or G·T (Δ) substrate (0.5 μM) in buffers of varying ionic strength. The rate was also determined at 0.16 M ionic strength (blue ▽) using 4-fold higher concentrations of TDG (20 μM) and G·T substrate (2 μM). Data are also shown for a 32 bp G·T substrate (0.5 μM) collected with 5 μM TDG (red ◇). (B) Equilibrium binding of TDG to G·T^F DNA substrate analog (10 nM) in buffers with ionic strength of 0.06 M (□), 0.11 M (○), 0.16 M (Δ), and 0.31 M (◇), monitored by fluorescence anisotropy (sulforhodamine labeled G·T^F DNA) at 22 °C. For each buffer, data from two independent binding experiments were fitted to a model with two binding sites for TDG (Materials and Methods), giving the following parameters: 0.06 M (□), $K_{\text{d1}} = 0.024 \pm 0.003$ μM , $K_{\text{d2}} = 0.54 \pm 0.09$ μM ; 0.11 M (○), $K_{\text{d1}} = 0.12 \pm 0.02$ μM , $K_{\text{d2}} = 0.98 \pm 0.2$ μM ; 0.16 M (Δ), $K_{\text{d1}} = 1.1 \pm 0.1$ μM , $K_{\text{d2}} = 10.5 \pm 1.3$ μM .

Fig. 4.

Temperature dependence of catalytic activity for TDG. Single turnover kinetics experiments were used to determine the rate of base excision for TDG (5 μM) as a function of temperature for G·U (○) and G·T (△) substrates (0.5 μM). We also determined the rate using 4-fold higher concentrations of TDG (20 μM) and G·T substrate (2 μM) at 10 °C (blue ▽). Data are also shown for a longer (32 bp) G·T substrate (0.5 μM) collected with 5 μM TDG (red ◇).

Fig. 5.

Thermal stability of TDG monitored by circular dichroism (CD) and fluorescence spectroscopy. (A) Effect of temperature on the structural integrity of TDG (2.5 μM) monitored by CD at 222 nm (CD_{222}). The increase in CD_{222} with temperature reflects heat-induced loss of secondary structure. As discussed in the text, thermal unfolding of TDG is irreversible, precluding a thermodynamic analysis (including T_m determination). (B) In addition to CD_{222} , the spectropolarimeter simultaneously records the photomultiplier (PM) voltage, which increases linearly with decreases in light intensity caused by absorption or light scattering. Because a temperature-dependent change in absorption is not expected (constant wavelength), changes in PM voltage are attributed to light scattering (turbidity) [44,45]. The initial increase in PM voltage is attributed to heat-induced aggregation of TDG, and the subsequent decrease is attributed to precipitation of aggregated protein. (C) Trp fluorescence spectra for TDG (1 μM) incubated at 37 $^{\circ}\text{C}$ for various lengths of time (as shown in key). Also shown is the spectrum for TDG incubated at 22 $^{\circ}\text{C}$ (dotted line), which does not change over time (for >2 hr). The increase in fluorescence intensity with time for TDG at 37 $^{\circ}\text{C}$ is attributed to light scattering arising from TDG aggregation. The intensity change is largely complete in 3 min, and occurs with a rate constant of 1 min^{-1} . (D) Trp fluorescence spectra of TDG (1 μM) at 37 $^{\circ}\text{C}$, in the presence of $\text{G}\cdot\text{T}^{\text{F}}$ analog (2 μM) or nonspecific DNA (NS28, 10 μM), collected after incubation times of 1 min or 120 min. Our previous studies show these DNA concentrations are saturating for these [28].