The Catalytic Power of Uracil DNA Glycosylase in the Opening of Thymine Base Pairs

Chunyang Cao, Yu Lin Jiang, Daniel J. Krosky, and James T. Stivers*

Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received April 28, 2006; E-mail: jstivers@jhmi.edu

Duplex DNA is a dynamic structure that shows both rapid unpairing of individual base pairs and larger cooperative unfolding events that occur on much slower time scales.[1,2] Since DNA is a ligand or substrate for a plethora of proteins and enzymes, it is of significant interest to understand the role of such dynamic fluctuations in site specific recognition. One paramount example is the role of spontaneous DNA base pair opening in the process of enzymatic recognition of damaged DNA bases by DNA glycosylases.[3] These DNA repair enzymes must locate extremely rare oxidized, deaminated, or alkylated bases in a large background of normal DNA bases in the genome that may be present in $>10^7$-fold excess over the damaged base.[3] In principle, two general mechanisms may be envisioned for location of such damaged sites: either the *site* itself possesses dynamic or structural features that lead to passive enzymatic recognition, or the *enzyme* possesses specialized search tools that allow for active inspection of the damaged base while its distinguishing structural features are still partially or completely obscured in the DNA base stack.

The DNA repair enzyme uracil DNA glycosylase (UNG) must find uracil bases in the genome that arise from spontaneous deamination of cytosine bases or the misincorporation of dUTP opposite to adenine during DNA replication.[3] It is well-established that UNG uses an extrahelical recognition mechanism where the uracil base and sugar are rotated 180° from the DNA base stack into a highly specific recognition pocket that excludes other natural DNA bases, including thymine and cytosine.[4,5] Thus, one question in uracil recognition is when along the base flipping reaction coordinate UNG discriminates between the structural congeners T and U. We have previously developed an NMR approach to address the question of whether UNG can transiently open TA base pairs and whether initial recognition involves spontaneous opening of the base pair to dock thymine in a loose recognition pocket that serves as a sieve to allow UNG to discriminate U from T[1]. These studies clearly revealed that *Escherichia coli* UNG specifically stabilizes thymine in an extrahelical state by slowing the closing rate. Most interestingly, UNG does this without increasing the rate of spontaneous TA base pair opening when it binds. The results were consistent with a passive mechanism of TA base pair inspection that is initiated by spontaneous, thermally induced motions of the thymine base that serve to expose it to a transient pyrimidine binding site on UNG.[6] Additional evidence that UNG can flip thymine is provided by the observation that the enzyme can be converted into a thymine DNA glycosylase by simply enlarging the active site such that the 5-methyl group is sterically accommodated.[7,8]

If a passive recognition model is generally applicable to UNG, then (i) the rate of thymine base pair opening in free and UNG-bound DNA should be equal for a series of thymine base pairs (TX) regardless of their thermal stability (Figure 1), and (ii) the closing rates in the presence of UNG should be similar even if the
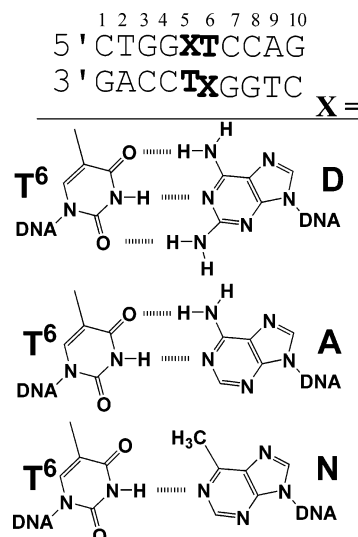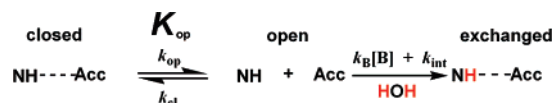

Figure 1. Sequence of palindromic 10mer duplexes containing isostructural T6X base pairs with one, two or three hydrogen bonds (D = diaminopurine, N = 6-methylpurine).

Scheme 1. DNA Base Pair Opening and Imino Proton Exchange[a]

[a] The constant k_{int} is the intrinsic rate for intramolecular catalysis of proton exchange by weakly basic N1 and N3 groups on A and C bases (ref 9). The chemical exchange rate is $k_{ex} = k_B[B] + k_{int}$.

closing rates differ widely in the free DNA. This is expected since, in a first approximation, the interaction of thymine with the UNG recognition site should be independent of the nature of the TX base pair. UNG is an ideal system for this analysis because it does not directly interact with the base opposing the uracil or thymine.[4,5]

To provide a rigorous test of these predictions we have now measured DNA base pair opening and closing rates in the absence and presence of UNG for a series of increasingly destabilized TX base pairs that contain one (T6N), two (T6A), or three (T6D) hydrogen bonds (Figure 1). Because the imino proton is protected from solvent while hydrogen bonded in a base pair, the base pair opening and closing rates (k_{op} and k_{cl}), and the equilibrium constant for opening (K_{op}) may be indirectly obtained by measuring the individual imino proton exchange rates using NMR solvent magnetization transfer methods (Scheme 1).[9] A powerful aspect of this approach is that the rate-limiting step for exchange may be changed from the chemical step (k_{ex}) to base pair opening (k_{op}) by addition of increasing concentrations of a proton exchange catalyst ($k_B[B]$).

Comparison of the measured opening rates (k_{op}) in Table 1 for each T6X base pair in the free and UNG-bound DNA shows that

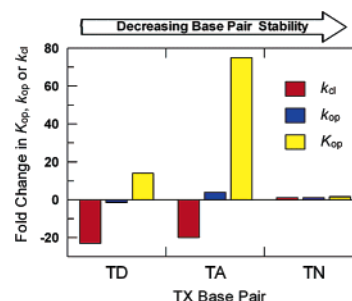**Table 1.** Base Pair Opening Parameters for the Thymine Base of the T^6X Base Pair in Free and UNG-Bound DNA[a]

TX base pair		αK_{op}[b] $(\times10^6)$	k_{op} (s^{-1})	k_{cl} $(\times10^{-6}\ s^{-1})$
T^6D	free	8.7 ± 0.5	61 ± 6	7 ± 2
	bound	120 ± 20	40 ± 10	0.3 ± 0.1
T^6A	free	20 ± 0.4	35 ± 6	1.8 ± 0.3
	bound	1500 ± 280	138 ± 20	0.09 ± 0.02
T^6N	free	500 ± 50	650 ± 200	1.3 ± 0.4
	bound	760 ± 100	700 ± 200	0.94 ± 0.2

[a] The parameters correspond to the mechanism in Scheme 1 and were obtained at pH 8.0, $T = 10\ °C$ using difluoroethylamine as a proton exchange catalyst[1]. The parameters for the TA base pair have been previously reported.[1] [b] The constant α takes into account possible steric effects of DNA that may hinder access of the general base catalyst to the imino site as compared to the free nucleoside.[9]

UNG does not actively accelerate base pair opening. This finding extends the previous conclusion to a series of isostructural base pairs with widely different thermodynamic stabilities[1]. Although the enzymatic opening rates show no consistent trend (modest decreases or a slight increase are observed, Table 1) the enzyme is found to slow the closing rates (k_{cl}) by factors of 23- and 20-fold for the T^6D (three H-bonds) and T^6A (two H-bonds) duplexes, but to have no effect on any exchange parameter for the destabilized T^6N base pair (one H-bond). In further experiments using a similar palindromic 10 mer DNA in which the central TA base pair was moved one position in the sequence (T^7A),[10] we have confirmed the general conclusion that UNG selectively promotes imino exchange of TA base pairs. However, as previously reported, imino proton exchange of guanine bases adjacent to TA pairs is also promoted by UNG, which is most reasonably attributed to a proximity effect because isolated GC pairs show no imino exchange enhancement with UNG.[1,10]

We note that the opening equilibrium (K_{op}) for the T^6X series in the free DNA increases by 55-fold in the order $T^6D < T^6A < T^6N$, and the trend is due mainly to increases in the opening rates. Two unexpected findings are that the opening rates for free T^6D and T^6A are similar despite the extra hydrogen bond in the T^6D pair and, furthermore, that free and bound T^6D show a 3-fold faster closing rate than the T^6A. A possible explanation for the similar opening rates is that opening of T^6D through the major groove only involves breaking of the hydrogen bonds involving the 6-NH_2 and N1 positions of D. Thus the third hydrogen bond between thymine O2 and the 2-NH_2 group of D serves as a pivot to partially hold the rotated T^6D pair in the stack and kinetically facilitate the closing rate. A similar conclusion was previously proposed in a computational study of GC base pair opening,[11] but more extensive experiments will be required to a establish a pivot mechanism for base pairs with three hydrogen bonds. UNG increases K_{op} for the T^6D and T^6A duplexes by 13 and 75-fold, but has no effect on K_{op} for T^6N. Thus, UNG does little work to assist opening of base pairs that are already kinetically and thermodynamically predisposed to open. This supports previous studies where it was found that uracil flipping by UNG is enhanced by weakly hydrogen bonded and flexible base pairs.[12,13]

The mechanistic implications of these data are best appreciated by comparison of the fold changes in the opening and closing parameters induced by UNG (Figure 2). The catalytic power of UNG in promoting imino proton exchange may be defined as the ratio $k_{op}^{UNG}/k_{op}^{free}$ or $k_{cl}^{UNG}/k_{cl}^{free}$ because increasing the opening rate (or decreasing the closing rate) promotes imino proton exchange (Scheme 1). The salient finding of Figure 2 is that UNG's catalytic power is attributed mostly to decreases in k_{cl}, whereas UNG has little catalytic power with respect to accelerating TX base pair

Figure 2. Relative effects of UNG on the equilibrium and kinetic parameters for T6:X base pair opening and closing. Negative numbers indicate UNG-induced decreases in a rate or equilibrium constant.

opening (k_{op}). These observations support a mechanism in which the very earliest event in recognition by UNG involves spontaneous expulsion of the base into a transient binding site that has been detected in crystallographic studies of the herpesvirus UNG bound to pTpTpT.[6,14] Once this site is transiently occupied, the base has an opportunity to partition forward along the base flipping reaction coordinate (in the case of uracil), or alternatively, to re-enter the DNA base stack (in the case of thymine). Since the lifetime of the extrahelical base in the free DNA is so short ($\sim$100 to 800 ns, Table 1), and UNG exhibits diffusion controlled binding kinetics, it is an improbable event that UNG encounters the extrahelical base during a bimolecular collision[1]. Instead, UNG must be already bound in the correct register to capture the pyrimidine base as it emerges from the duplex, providing a mechanistic role for short-range sliding on duplex DNA.[3,5] The universality of such a sieve mechanism is not yet clear, because crystallographic studies on MutM, a glycosylase that removes oxidized guanine residues (G°), suggested that it actively destabilizes GC and G°C base pairs using a Phe side chain, but only flips G°.[15] In contrast, a sieving site that binds both extrahelical G and G° has been proposed with another 8-oxoguanine DNA glycosylase (hOgg1).[16] In any event, solution NMR has allowed detection of a high energy state important for UNG recognition, and this state would be difficult or impossible to uncover using any other method.

Supporting Information Available: Experimental details, synthesis of 6-methylpurine phosphoramidite, NMR spectra, exchange time courses, buffer dependence of exchange rates, and table reporting the exchange parameters for all imino protons in each TX duplex. This material is available free of charge via the Internet at http://pubs.acs.org.

References
(1) Cao, C.; Jiang, Y. L.; Stivers, J. T.; Song, F. *Nat. Struct. Mol. Biol.* **2004**, *11*, 1230.
(2) Parker, C. N.; Halford, S. E. *Cell* **1991**, *66*, 781.
(3) Stivers, J. T.; Jiang, Y. L. *Chem. Rev.* **2003**, *103*, 2729.
(4) Parikh, S. S.; Walcher, G.; Jones, G. D.; Slupphaug, G.; Krokan, H. E.; Blackburn, G. M.; Tainer, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5083.
(5) Stivers, J. T. *Prog. Nucleic Acid Res. Mol. Biol.* **2004**, *77*, 37.
(6) Savva, R.; McAuley-Hecht, K.; Brown, T.; Pearl, L. *Nature* **1995**, *373*, 487.
(7) Kwon, K.; Jiang, Y.; Stivers, J. *Chem. Biol.* **2003**, *10*, 1.
(8) Kavli, B.; Slupphaug, G.; Mol, C. D.; Arvai, A. S.; Peterson, S. B.; Tainer, J. A.; Krokan, H. E. *EMBO J.* **1996**, *15*, 3442.
(9) Gueron, M.; Leroy, J.-L. *Methods Enzymol.* **1995**, *261*, 383.
(10) See Supporting Information.
(11) Giudice, E.; Varnai, P.; Lavery, R. *Nucleic Acids Res.* **2003**, *31*, 1434.
(12) Krosky, D. J.; Schwarz, F. P.; Stivers, J. T. *Biochemistry* **2004**, *43*, 4188.
(13) Krosky, D. J.; Song, F.; Stivers, J. T. *Biochemistry* **2005**, *44*, 5949.
(14) Jiang, Y. L.; Krosky, D. J.; Seiple, L.; Stivers, J. T. *J. Am. Chem. Soc.* **2005**, *127*, 17412.
(15) Banerjee, A.; Santos, W. L.; Verdine, G. L. *Science* **2006**, *311*, 1153.
(16) Banerjee, A.; Yang, W.; Karplus, M.; Verdine, G. L. *Nature* **2005**, *434*, 612.

JA062978N