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Linear Free Energy Correlations for Enzymatic Base Flipping: How Do Damaged Base Pairs Facilitate Specific Recognition?[†]

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ABSTRACT: To efficiently maintain their genomic integrity, DNA repair glycosylases must exhibit high catalytic specificity for their cognate damaged bases using an extrahelical recognition mechanism. One possible contribution to specificity is the weak base pairing and inherent instability of damaged sites which may lead to increased extrahelicity of the damaged base and enhanced recognition of these sites. This model predicts that the binding affinity of the enzyme should increase as the thermodynamic stability of the lesion base pair decreases, because less work is required to extrude the base into its active site. We have tested this hypothesis with uracil DNA glycosylase (UDG) by constructing a series of DNA duplexes containing a single uracil (U) opposite a variety of bases (X) that formed from zero to three hydrogen bonds with U. Linear free energy (LFE) relationships were observed that correlated UDG binding affinity with the entropy and enthalpy of duplex melting, and the dynamic accessibility of the damaged site to chemical oxidation. These LFEs indicate that the increased conformational freedom of the damaged site brought about by enthalpic destabilization of the base pair promotes the formation of extrahelical states that enhance specific recognition by as much as 3000-fold. However, given the small stability differences between normal base pairs and U•A or U•G base pairs, relative base pair stability contributes little to the $>10^6$-fold discrimination of UDG for uracil sites in cellular DNA. In contrast, the intrinsic instability of other more egregious DNA lesions may contribute significantly to the specificity of other DNA repair enzymes that bind to extrahelical bases.

The genetic information of a cell can be irreversibly altered through the chemical modification of nucleotide bases (*1*). To combat these mutagenic effects, organisms have evolved a two-tiered base excision repair (BER) pathway that handles a wide array of base lesions (*2*). In the first stage, a highly specific DNA repair glycosylase excises the damaged base from the DNA (*3*, *4*), producing an abasic site. This intermediate is then processed by the sequential action of several repair enzymes that ultimately restore the site to its original state (*2*). Since damaged site specificity resides solely with the DNA glycosylase, these enzymes must possess extraordinarily high catalytic specificities (*4*, *5*). In the absence of such specificity, undamaged bases would be randomly excised from DNA, leading to undesirable abasic sites and genetic instability (*6*−*10*).

In general, enzymatic specificity results from the extraordinary structure of enzyme active sites that disfavors formation of catalytically productive interactions with non-substrate molecules, and strongly favors such interactions with the true substrates. As an essential part of their recognition mechanisms, all DNA glycosylases extrude their damaged bases from the DNA double helix in a process known as base flipping, thereby placing it extrahelically into their active sites where specific interactions with the damaged base can be formed (*11*, *12*). As part of the energetic cost of base flipping, the hydrogen bonds and stacking interactions of the base pair must be disrupted. Thus, a prediction is that DNA glycosylases should bind more tightly to damaged sites with disrupted base pairing because it requires less binding energy to flip the damaged base from the destabilized site (*4*, *13*−*22*). Such a thermodynamic mechanism is quite general, and would apply even for DNA glycosylases that interact with the base that opposes the damaged base (*23*−*25*).

How much does the intrinsic thermodynamic stability of the damaged base pair contribute to specific damaged site binding by DNA glycosylases? We have investigated this question using the enzyme uracil DNA glycosylase (UDG),[1] which removes uracil from U•G and U•A base pairs in duplex DNA (*14*, *26*, *27*). The approach was to measure the binding affinity of UDG for a series of DNA duplexes, in which the number of hydrogen bonds (*n*) between uracil and its opposing base (X) were systematically varied (Figure 1A).

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[1] Abbreviations: UDG, uracil DNA glycosylase; U^F, 2′-β-fluoro-2′-deoxyuridine; ϕ, abasic site; D, 2,6-diaminopurine; M, 4-methylindole; N, nebularine; DSC, differential scanning calorimetry; ψ, pseudo-dihedral angle.

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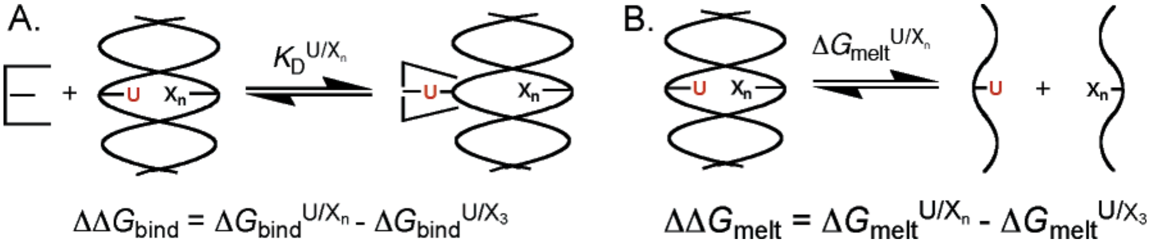

FIGURE 1: (A) Specific binding of UDG (E) to a DNA duplex containing deoxyuridine (U) opposite a purine analogue (X). In this study, the number of hydrogen bonds (n) in the U•X_n base pair varies from zero to three. Since binding of uracil requires breaking of the base pair hydrogen bonds, the difference in binding free energy ($\Delta\Delta G_{bind}$) between a DNA duplex with n hydrogen bonds and a reference duplex with three hydrogen bonds (U•X_3) should in part reflect the reduced energetic cost of breaking the hydrogen bonds in the base pair. (B) Dissociation of a duplex with a U•X_n base pair into two single strands. The difference in the duplex melting free energy ($\Delta\Delta G_{melt}$) between a U•X_n duplex and the reference duplex (U•X_3) will also reflect the energetic effects of a destabilized base pair.

Then, these binding affinities were correlated with rigorous measurements of the thermodynamic stabilities of these duplexes (Figure 1B). UDG was selected for this study because, unlike many DNA glycosylases (*23*−*25*), it does not make any direct contacts with the base opposite uracil (*11*). Thus, the observed changes in binding affinity can be largely attributed to the relative stability of the U•X_n base pairs in the free duplex DNA and not differential interactions of the enzyme with the various opposing bases. These quantitative free energy correlations provide the first direct evidence that promotion of extrahelical conformations by enthalpic destabilization of a damaged site can indeed enhance the specific binding of a DNA repair enzyme.

EXPERIMENTAL PROCEDURES[2]

Materials. The 2′-deoxynucleoside phosphoramidites, CPG supports, and DNA synthesis reagents were purchased from Glen Research (Sterling, VA), except for 2′-β-fluoro-2′-deoxyuridine (U^F), which was synthesized as described previously (*5*, *28*). The oligonucleotides were synthesized using standard phosphoramidite chemistry on an Applied Biosystems 392 synthesizer. The oligonucleotides were purified by anion exchange HPLC (Zorbax), followed by C-18 reversed phase HPLC (Phenomenex Aqua column). Fractions containing pure oligonucleotide were concentrated to dryness *in vacuo*, redissolved in MilliQ water, and stored at −20 °C. The purity of the oligonucleotides was assessed by matrix-assisted laser desorption mass spectroscopy and denaturing polyacrylamide gel electrophoresis. The concentration of each oligonucleotide was determined using its extinction coefficient at 260 nm (*29*). DNA duplexes were hybridized in 10 mM Tris-HCl (pH 8.0) and 25 mM NaCl as described previously (*5*). The purification of *Escherichia coli* UDG has been described previously (*30*).

K_D Measurements. The K_D values for binding of the U^F•X_n duplexes to UDG were measured essentially as described using a kinetic competitive inhibition HPLC assay under conditions where the apparent K_i is equal to the K_D value (i.e., $[S] \ll K_m$, where S exhibits rapid equilibrium binding) (*31*). The only modification was that the abasic product (AΦAp) and reactant (AUAp) were separated using isocratic conditions with 9.5% CH_3CN and 0.1 M triethylammonium acetate. Reaction mixtures (35 μL) containing 10 mM Tris-HCl (pH 8.0), 60 mM NaCl, 12.5 μg/mL BSA, 1 μM AUAp, 0.5 nM UDG, and a variable amount of the U^F•X_n duplex were incubated at room temperature for 5 min. The K_D for each duplex was determined by fitting to eq 1

$$k_i/k_o = 1/(1 + [U^F{\bullet}X_n]/K_D) \qquad (1)$$

where k_i is the inhibited rate and k_o is the rate in the absence of competitor DNA. For the tightest binding duplex (U^F•M), eq 1 was modified to take into account inhibitor depletion (*32*).

The differences in binding free energies relative to the duplex with three hydrogen bonds (U^F•D) were calculated from the measured K_D values using eq 2.

$$\Delta\Delta G_{bind} = RT \ln[K_D(U^F{\bullet}X_n)/K_D(U^F{\bullet}D)] \qquad (2)$$

Fluorescence Spectroscopy. To ascertain that all of the DNA duplexes attained the same bound state, tryptophan fluorescence measurements of free and DNA-bound UDG were performed. Samples (497 μL) containing 10 mM Tris-HCl (pH 8.0), 60 mM NaCl, and 300 nM UDG were incubated for 3 min at 25 °C in a 10 mm quartz cuvette, and a fluorescence emission spectrum was recorded in the range of 325−425 nm on a SPEX FluoroMax-3 fluorimeter ($\lambda_{ex} = 295$ nm). U^F•X_n duplex DNA (2.5 μL) was then added to the UDG solution to give a final DNA concentration of 500 nM. The reaction mixture was magnetically stirred and incubated for 3 min at 25 °C, before the fluorescence emission spectrum of the UDG•DNA complex was recorded. The tryptophan fluorescence intensities of free and DNA-bound UDG at 333 nm were measured, and the raw values were then normalized for the fraction of UDG bound to each DNA analogue before the ratio ($F^{333}{}_{bound}/F^{333}{}_{free}$) was calculated.

Differential Scanning Calorimetry. DSC measurements of duplex strand melting were taken using a VP-DSC microcalorimeter from Microcal, Inc. (Northampton, MA) essentially as described previously (*33*). The DNA solutions had a concentration of 20 μM in DNA duplex with 10 mM Na_2HPO_4 (pH 7.5) and 60 mM NaCl. Samples were equilibrated at 20 °C for 15 min and scanned up to 95 °C at a preset scan rate of 60 °C/h. The transition peak areas were measured using the EXAM software program (*34*), and the transition peak areas were divided by the DNA duplex concentration to provide the transition enthalpies. Transition

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[2] Certain commercial materials, instruments, and equipment are identified herein to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material, instruments, or equipment identified is necessarily the best available for the purpose.

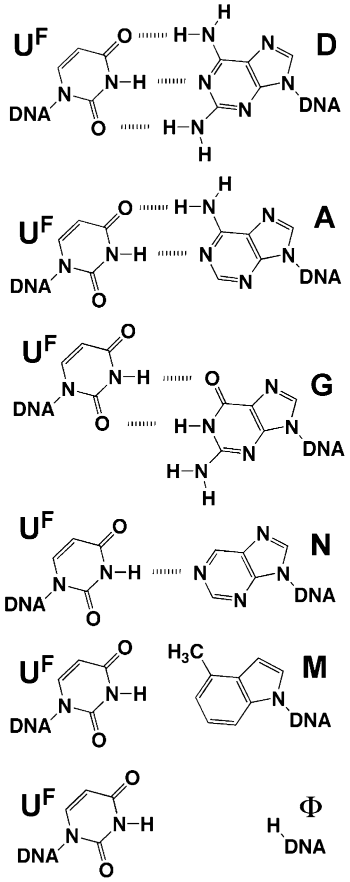

FIGURE 2: Structures of $U^F\cdot X_n$ base pairs: $U^F\cdot D$ (*54*, *55*), $U^F\cdot G$ (*56*), $U^F\cdot N$ (*57*, *58*), $U^F\cdot\Phi$ (*59*−*61*), and $U^F\cdot M$ (*62*).

entropies were determined from plots of C_p/T against T, by dividing the transition peak area by the DNA duplex concentration. Procedures for buffer baseline corrections and accounting for pre- and post-transition baselines have been described previously (*33*).

$KMnO_4$ Oxidation Measurements. Because of the low reactivity of U^F to oxidation (*35*), it was replaced with thymine (T) in the oligonucleotides used in this study. To reaction mixtures (20 μL) containing 10 mM Tris-HCl (pH 8.0), 60 mM NaCl, and either 100 nM single-stranded 5′-$[^{32}P]$T or 5′-$[^{32}P]T\cdot X_n$ duplex was added 2.5 mM $KMnO_4$. After the sample had been incubated for 3 min at room temperature, the reaction was halted by the addition of 20 μL of a stop solution containing 1.5 M sodium acetate, 1 M 2-mercaptoethanol, and 200 μg/mL tRNA. The samples were processed, imaged, and quantified as described previously (*32*).

RESULTS

Binding of UDG to Destabilized Damaged Sites. A series of 15mer duplexes were constructed in which 2′-β-fluoro-2′-deoxyuridine (U^F), a nonhydrolyzable uracil analogue (*5*), was placed opposite a series of bases (X) which form zero to three hydrogen bonds with U^F (Figure 2). The affinity of UDG for each $U^F\cdot X$ duplex was measured by a competitive inhibition kinetic assay in which a 3mer substrate (AUAp) is separated from the abasic product (AΦAp) using reverse phase HPLC (Figure 3A) (*31*). Representative inhibition data are shown in Figure 3B for the duplex that contains a uracil•4-methylindole base pair ($U^F\cdot M$). The K_D values for the six duplexes showed an incremental decrease as the number of hydrogen bonds was ablated (Figure 4A). The tightest observed binding affinity was for the $U^F\cdot M$ construct that has no hydrogen bonds (K_D = 0.25 nM). This affinity is 3000-fold tighter than that of the uracil•diaminopurine duplex that has three hydrogen bonds ($U^F\cdot D$, K_D = 820 nM). Similar tight binding was observed for the duplex with an abasic site opposite U^F ($U^F\cdot\Phi$, K_D = 2.4 nM), providing additional evidence that complete ablation of hydrogen bonding leads to a significantly increased binding affinity.[3]

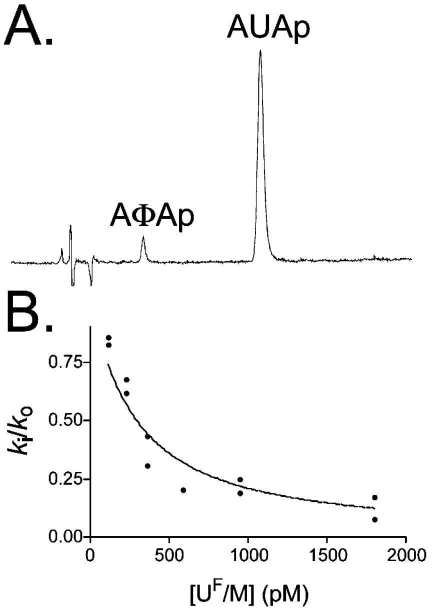

FIGURE 3: Determination of the binding affinity of specific DNA duplexes using a competitive inhibition assay. (A) UDG HPLC activity assay. The substrate (AUAp) and product AΦAp are indicated. (B) Inhibition of UDG by a U•M duplex (K_D = 0.24 ± 0.03 nM).

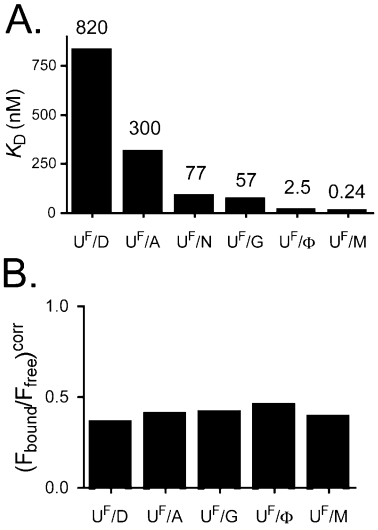

FIGURE 4: (A) Binding affinities of UDG for $U^F\cdot X_n$ DNA duplexes. The K_D values (nanomolar) are shown above the individual bars. (B) Quench of UDG tryptophan fluorescence upon binding of the $U^F\cdot X_n$ duplex. The degree of UDG quenching was normalized to reflect the quenching when UDG is saturated with DNA (see Experimental Procedures).

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[3] The observed K_D for the $U^F\cdot\Phi$ duplex (2.5 nM) predominantly reflects binding of UDG to U^F, and not to Φ, because the K_D for abasic DNA is much weaker (70 nM for T•Φ and 100 nM for Φ•A).

Table 1: Thermodynamic Parameters for UDG Binding, DNA Melting, and Permanganate Accessibility of Destabilized Base Pairs

duplex	K_D (nM)	ΔG_{bind} (kcal/mol)	ΔH_{melt} (kcal/mol)	ΔS_{melt} (cal mol^{-1} K^{-1})	ΔG_{melt}[a] (kcal/mol)	log S[b]
$U^F \cdot D$	820 ± 90	−8.3 ± 0.1	58.9 ± 1.8	0.16 ± 0.01	10.9 ± 2.3	−1.4 ± 0.14
$U^F \cdot A$	300 ± 50	−8.9 ± 0.1	39.6 ± 4.0	0.11 ± 0.01	7.1 ± 5.2	−1.0 ± 0.06
$U^F \cdot N$	77 ± 11	−9.7 ± 0.1	40.5 ± 2.0	0.10 ± 0.01	9.2 ± 2.5	ND[c]
$U^F \cdot G$	57 ± 6	−9.9 ± 0.1	34.0 ± 2.4	0.10 ± 0.01	5.6 ± 3.2	−0.80 ± 0.03
$U^F \cdot \Phi^3$	2.5 ± 0.4	−11.7 ± 0.1	4.3 ± 0.1	0.01 ± 0.01	0.3 ± 0.3	−0.22 ± 0.01
$U^F \cdot M$	0.24 ± 0.03	−13.1 ± 0.1	ND[c]	ND[c]	ND[c]	−0.36 ± 0.01

[a] Calculated at 298 K. [b] S is the relative sensitivity of a T•X base pair to oxidation by $KMnO_4$ (see the legend of Figure 6). [c] Not determined.

These results indicate that removal of three hydrogen bonds can enhance specific recognition by up to 4.8 kcal/mol.

In large part, these binding effects reflect the thermodynamic properties of the free damaged site because these discrete base pair perturbations are not expected to affect interactions between UDG and the DNA.[4] This conclusion is supported by inspection of the crystal structures of UDG complexed with substrate analogues, which show that UDG does not make any interactions with the base that opposes the damage site, or with the undamaged strand (*36*). Thus, recognition solely involves the extrahelical deoxyuridine and not other specific features of the base pair or duplex. To further establish that all of the $U^F \cdot X$ duplexes used here attain the same bound state, and that the observed effects on binding largely arise from the properties of the free DNA, we measured the tryptophan fluorescence quenching upon binding of each duplex (Figure 4B). Previous work has shown that the quenching of UDG tryptophan fluorescence upon specific DNA binding is a sensitive measure of an induced fit conformational change in UDG that is required to achieve the final productive conformation with a flipped-out uracil (*5*). Within the errors of these measurements, all of the duplexes produced the same magnitude of fluorescence quenching, indicating that the same bound conformations were attained for all.

Thermodynamic Stabilities of $U^F \cdot X$ Duplexes. We then determined the energetic effects of this series of site-specific base pair disruptions on the thermodynamic parameters for DNA melting using differential scanning calorimetry (DSC, Figure 5). With DSC, one can measure the enthalpy (ΔH_{melt}) and entropy (ΔS_{melt}) of the melting transition directly, and unlike optical methods, it is insensitive to the mechanism of duplex melting (*37*). The free energy of duplex dissociation (ΔG_{melt}) at any temperature can then be simply calculated from ΔH_{melt} and ΔS_{melt}, using the relationship $\Delta G_{melt} = \Delta H_{melt} - T\Delta S_{melt}$, because the heat capacity of the duplex and that of the single strands are equal ($\Delta C_p \approx 0$) (*38, 39*). As expected, $U^F \cdot X$ duplexes exhibited decreasing transition enthalpies in the ΔH_{melt} range of 58.9−4.3 kcal/mol as the number of hydrogen bonds was decreased. The complete thermodynamic parameters for duplex melting are reported in Table 1.

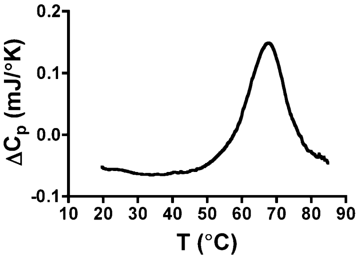

FIGURE 5: Differential scanning calorimetry (DSC) of the $U^F \cdot D$ duplex. A 20 μM DNA duplex solution in 10 mM sodium phosphate (pH 7.5) and 60 mM NaCl was placed inside of the DSC sample cell, and the change in the heat capacity of the solution (ΔC_p) was monitored as it was warmed from 20 to 95 °C at a rate of 60 °C/h. The thermodynamic parameters of duplex dissociation were extracted as described in Experimental Procedures.

Correlation of UDG Binding Affinity with Damaged Base Pair Stability. To quantitatively evaluate the impact of damaged base pair disruption on UDG affinity, the differences in binding free energies ($\Delta\Delta G_{bind}$, Figure 1) were plotted against the changes in transition enthalpies ($\Delta\Delta H_{melt}$) and entropies ($-T\Delta\Delta S_{melt}$) (Figure 6A,B). In this analysis, the difference energies are relative to the duplex with three hydrogen bonds ($U^F \cdot D$), and the value of $-T\Delta\Delta S_{melt}$ was calculated at 298 K. $\Delta\Delta G_{bind}$ was found to increase linearly as the transition enthalpy and entropy decrease: $\Delta\Delta H_{melt}$ (slope $= 0.064 \pm 0.01$, $r^2 = 0.937$) and $-T\Delta\Delta S_{melt}$ (slope $= 0.080 \pm 0.011$, $r^2 = 0.949$). As required from these linear correlations, a strong correlation with $\Delta\Delta G_{melt}$ (slope $= 0.3 \pm 0.1$, $r^2 = 0.839$) was also observed (data not shown). The implications of these correlations are discussed below.

Correlation of UDG Binding Affinity with Damaged Base Accessibility. The above thermodynamic correlations suggest that these destabilized duplexes might exhibit an increase in the number of dynamic fluctuations that promote extrahelical states of U^F at temperatures well below the duplex melting temperature. Since the dynamic accessibility of the damaged base is another factor that could enhance its recognition by DNA glycosylases, it was of interest to measure the relative accessibility of each destabilized base pair, and correlate this parameter with UDG binding affinity.

To explore this question, a potassium permanganate ($KMnO_4$) sensitivity assay was employed (*32*, *35*, *40*). Since sites of pyrimidine oxidation are susceptible to strand cleavage under basic conditions, they can be detected as fragments using polyacrylamide gel electrophoresis (Figure 7A) (*41*). In these studies, U^F was first replaced with thymine (T) because of its poor oxidation reactivity arising from its electron deficient 5,6-double bond (data not shown). This is a very conservative change, as a T•X base pair will have base pairing strength and geometry nearly identical to those of a $U^F \cdot X$ base pair (*42*, *43*). As shown in Figure 7A, decreasing the base pair strength leads to an increase in the sensitivity of the T•X base pair to permanganate oxidation, and also the invariant T•A base pair three nucleotides away,

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[4] The observation of linear free energy relationships between the thermodynamic parameters of the free duplex DNA and the overall free energy of DNA binding is not negated if different energetic interactions exist between the enzyme and each bound duplex. However, the observed slopes will reflect the relative effects of perturbing the duplex in the free and bound state.

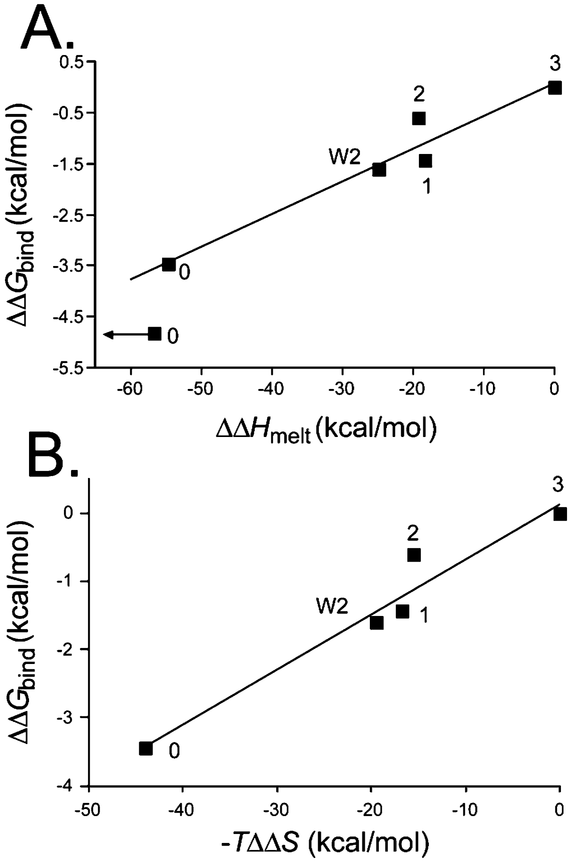

FIGURE 6: Correlations between UDG binding affinity for each $U^F{\cdot}X_n$ duplex ($\Delta\Delta G_{bind}$) and the thermodynamic parameters for duplex melting. The numbers of hydrogen bonds in the $U^F{\cdot}X_n$ base pair are indicated. (A) $\Delta\Delta G_{bind}$ vs $\Delta\Delta H_{melt}$ (slope $= 0.064$, $r^2 =$ 0.937). Only an upper limit for the enthalpy of melting for the $U^F{\cdot}$M duplex was obtained (arrow). (B) $\Delta\Delta G_{bind}$ vs $-T\Delta\Delta S_{melt}$ (slope $= 0.080$, $r^2 = 0.949$).

indicating that even very conservative changes to the T·X base pair can influence the dynamics of neighboring base pairs in the duplex (*44*). A plot of log K_D against log(relative $KMnO_4$ sensitivity) shows a linear correlation (Figure 7B), establishing that increasing base accessibility at temperatures well below the T_m value has a strong positive effect on binding affinity. As an important control, the amount of oxidized product was found to increase linearly with respect to time and concentration of $KMnO_4$ (data not shown). Therefore, the differences in the sensitivity of these duplexes to oxidation directly reflect the unfavorable dynamic pre-equilibrium for exposure of the thymidine base prior to reaction with $KMnO_4$.

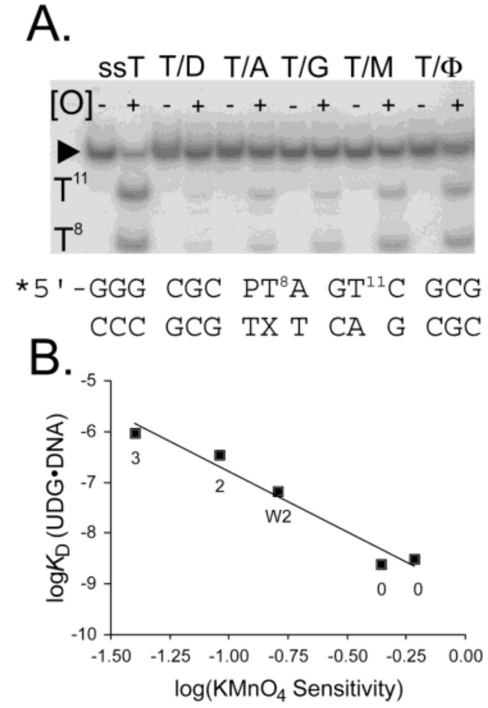

FIGURE 7: (A) Sensitivity of thymine in single-stranded and duplex DNA to oxidation by potassium permanganate. Samples of 5′-^{32}P-labeled single-stranded or $T{\cdot}X_n$ duplex DNAs were reacted with 2.5 mM $KMnO_4$ ([O]) for 3 min. After the oxidized strands had been cleaved with piperidine, the reaction mixtures were run a 19% denaturing polyacrylamide gel, and the radioactivity of each band was quantified with a phosphorimager. The position on the gel of the full-length DNA is marked with a black wedge. (B) Correlation between UDG binding affinity and $KMnO_4$ sensitivity (slope $=$ -2.37 ± 0.270, $r^2 = 0.963$). The relative sensitivity of a thymine in a $T{\cdot}X_n$ base pair is defined as $[(I^{T8} - I^{bkgd})/(I^{total} - I^{bkgd})^{duplex} \times 100]/[(I^{T8} - I^{bkgd})/(I^{total} - I^{bkgd})^{single-stranded} \times 100]$, where I^{T8} is the intensity of the band corresponding to oxidation of the T in the $T{\cdot}X_n$ base pair, I^{bkgd} is the background correction, and I^{total} is the sum of all of the intensities of the bands in a given lane. The numbers of hydrogen bonds in the $T{\cdot}X_n$ duplex are indicated.

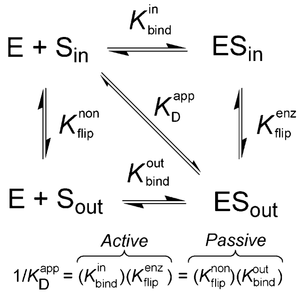

FIGURE 8: Thermodynamic model showing the energetic equivalence of the passive and active base flipping mechanisms (see the text). The equivalence of the pathways is illustrated by the thermodynamic box, which requires that $1/K_D^{app} = K_{in}^{bind}K^{enz}_{flip} = K^{non}_{flip}K_{out}^{bind}$.

DISCUSSION

Thermodynamic Framework for Active and Passive Base Flipping. While it is clear from structural and spectroscopic studies that DNA glycosylases bind their cognate damaged base in an extrahelical conformation (*3*, *11*), the pathway by which the damaged base is flipped out of the DNA duplex and placed inside of the enzyme active site remains poorly defined. In one model, DNA glycosylases *passively* capture damaged bases that are transiently extrahelical (Figure 8) (*14*, *45*). According to this view, the DNA glycosylase does not lower the activation energy or equilibrium for damaged base flipping, but instead relies on the increased extrahelical propensity of damaged bases to enhance bimolecular encounter. In the alternative view, DNA glycosylases *actively* flip out their cognate lesions by destabilizing the damaged site in an initial encounter complex. Active base flipping may occur by stabilization of high-energy intermediate conformations on the base flipping pathway, or by the use of mechanical forces to propel the base from the duplex (Figure 8) (*5*, *46*). It is important to point out that passive and active flipping pathways cannot be distinguished by *thermodynamic measurements alone*. This conclusion is required because $K_{in}^{bind}K^{enz}_{flip} = K^{non}_{flip}K_{out}^{bind}$, as shown in

Figure 8. Only an assessment of the *kinetic competence* of each pathway can reveal whether passive or active base flipping is the major route taken for a given system.

Specific Recognition and Conformational Freedom of Damaged Sites. We have found that the affinity of UDG for a specific site is linearly dependent on the enthalpy of duplex dissociation (Figure 6A). The linear relationship between UDG affinity and ΔH_{melt} confirms the notion that weakened base pairing decreases the enthalpy of duplex melting and, consequently, increases UDG binding affinity (Figure 6A). However, this finding alone provides little insight into the physical mechanism by which decreases in base pair enthalpy lead to enhanced binding. The small slope of the correlation suggests that the higher affinity of UDG for destabilized damaged sites does not simply arise from the reduced enthalpic cost of breaking a destabilized base pair during base flipping, and that other energetic influences must be at work.[4] It should be stated that the experiments presented here are explicitly designed to probe the enthalpic and entropic contributions of the damaged site alone to specific recognition. The total enthalpy and entropy of binding (including the enzyme, DNA, and solvent) are not evaluated in any of the current experiments, and in fact, these measurements are not required for the conclusions presented below.

The plot of $\Delta\Delta G_{\mathrm{bind}}$ versus $-T\Delta\Delta S_{\mathrm{melt}}$ reveals that there is an equally significant correlation between changes in duplex entropy and binding affinity (Figure 6B). The parameter $-T\Delta\Delta S_{\mathrm{melt}}$ likely reflects the increased conformational flexibility of the destabilized base pairs in the duplex DNA, because the entropy differences of the dissociated single-stranded DNAs in the melting experiments should be similar, given the conservative changes in these substrates. If we assume this physical interpretation for the entropy changes between these DNA constructs, the correlation suggests that increased flexibility of the base pair produces conformational states that are productive for UDG binding. The conclusion that extrahelical conformational states are produced is supported by the correlation between UDG binding affinity and $\mathrm{KMnO_4}$ sensitivity (Figure 7B), which reflects the dynamic equilibrium of the base between an inaccessible and permanganate accessible state (i.e., an extrahelical exposed conformation). A reasonable interpretation of these combined findings is that enthalpic destabilization of the base pair allows increased conformational flexibility, producing extrahelical conformers, some of which favor enzyme binding.

The effects of base pair enthalpy and entropy on the extrahelical conformational distributions that may promote base flipping are depicted in Figure 9A−C. In these panels, the probability of an extrahelical conformation is plotted against the backbone pseudodihedral angle (ψ) of the deoxyuridine nucleotide, defined as indicated in Figure 9. The angle ψ has been previously used in computational studies to describe the pathway for base flipping (*47*), and is used here because of its simple representation of the base flipping trajectory, although none of the arguments depend on this formalism. Using this nomenclature, a ψ of 10° reflects the fully base paired state and a ψ of 180° reflects the fully extrahelical state. In free DNA (Figure 9A), the U nucleotide in the stable base pair with three hydrogen bonds (U•D) should be tightly centered around an average conformation with a ψ of 10° (red curve), while the unstable construct with no hydrogen bonds (U•Φ) should be broadly centered around a ψ value further along the flipping reaction coordinate (green curve). Thus, enthalpic destabilization of the base pair in a passive mechanism leads to conformations that facilitate binding of the enzyme (see Figure 8, counterclockwise pathway).

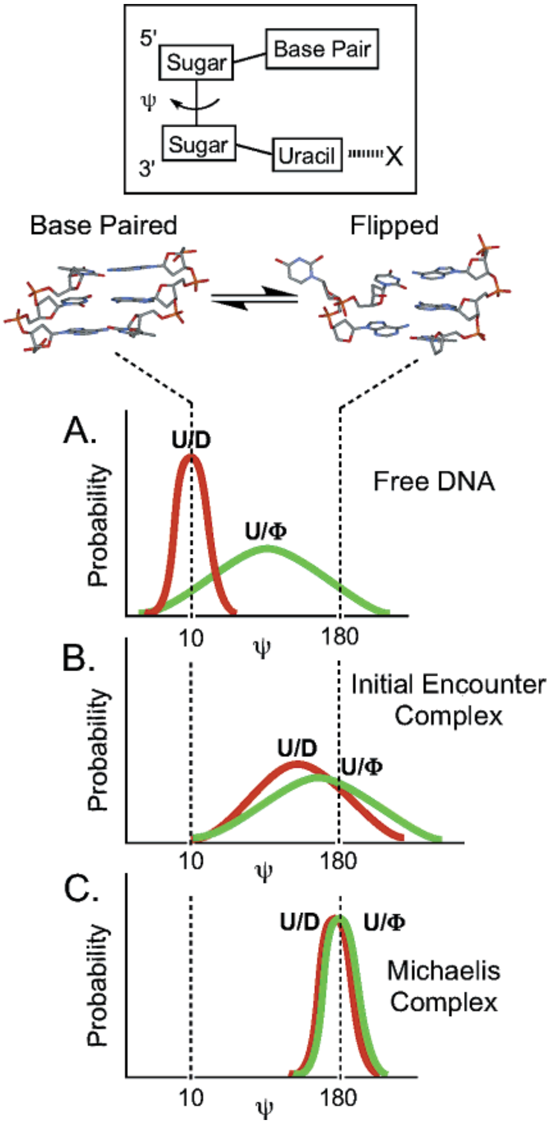

FIGURE 9: Enthalpic destabilization of the $\mathrm{U{\bullet}X}_n$ base pair leads to extrahelical conformations that promote binding. The probability of an extrahelical conformer as a function of the pseudodihedral angle ψ, which is a measure of the progress along the base flipping reaction coordinate, is indicated (see the text and ref *47*). Using this nomenclature, a fully stacked base pair has a ψ value of ~10°, whereas the fully extrahelical conformation has a ψ value of 180°. (A) Enthalpic destabilization of the base pair in the free DNA leads to extrahelical conformations that promote *passive* base flipping. (B) Base pair destabilization can also affect an active mechanism in which UDG forms an initial encounter complex with the DNA in which the base is not yet fully extrahelical (*5*, *46*, *48*). In this case, enzyme binding energy is used to destabilize the U•D base pair, allowing it to achieve extrahelical states already available to the U•Φ duplex due to its intrinsic instability. (C) A hypothetical distribution of extrahelical conformers for the final UDG•DNA complex. The enzyme has fully stabilized the flipped-out uracil, and the distribution of conformational states for the U•D and U•Φ duplexes is narrowly focused around a ψ of 180° (*36*, *47*).

For the alternative active base flipping mechanism (Figure 9B), binding of UDG in an initial encounter complex can alter the DNA structure such that the more stable U•D base pair is also destabilized (Figure 9B, red curve) (*5*, *46*, *48*). In this initial destabilized complex, the partially extrahelical uracil may assume a similar average conformation, and broad distribution, for both the U•D and U•Φ constructs. However, flipping of U in the U•D construct requires a greater amount of binding energy to overcome the enthalpic barrier to base flipping, resulting in weaker binding of the U•D duplex compared to that of the U•Φ duplex. Finally, in the Michaelis complex, in which the base is fully extrahelical (Figure 9C), both the U•D and U•Φ duplexes assume the same average conformation and small conformational distribution which are enforced by the strong interactions between the enzyme and uracil (*36*, *46*). Although this mechanism implies a reduction in the conformational flexibility of destabilized base pairs such as the U•Φ duplex upon formation of the Michaelis complex, this expected unfavorable entropic contribution to the overall free energy of binding may be paid for by the even larger enthalpic benefit of base pair destabilization for such conformationally flexible substrates (Table 1).

CONCLUSIONS

We have shown that the maximum gain in specific recognition by UDG arising from destabilization of the damaged base pair is 4.8 kcal/mol, and that this energetic effect likely arises from increasing the population of extrahelical states that promote binding. Since the catalytic specificity of UDG for uracil as opposed to other normal bases has been estimated to be at least 8.3 kcal/mol *in vitro* (*5*), the energetic contribution of destabilized damaged sites to specific ground state binding can be significant, at least in this model system. However, the *in vivo* substrates of UDG consist of U•A or U•G base pairs, which are not significantly destabilized compared to other normal base pairs. Thus, spontaneous base flipping at damaged sites is not a viable mechanism for accounting for the specificity of UDG *in vivo*. The remaining specificity of UDG must be attributed to strong transition state interactions that can be induced only by actively flipping the uracil base into the active site (*5*, *49*). Although unimportant for UDG, damaged site instability could contribute significantly to specific recognition by repair enzymes that act on intrinsically unstable base pairs such as O^6-methylguanine (*45*), hypoxanthine (*50*, *51*), N^1-methyladenine, and N^3-methylcytosine (*52*, *53*).

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