Energetics of Lesion Recognition by a DNA Repair Protein: Thermodynamic Characterization of Formamidopyrimidine-glycosylase (Fpg) Interactions with Damaged DNA Duplexes

ARTICLE *in* JOURNAL OF MOLECULAR BIOLOGY · JUNE 2003

Impact Factor: 4.33 · DOI: 10.1016/S0022-2836(03)00365-6 · Source: PubMed

doi:10.1016/S0022-2836(03)00365-6 *J. Mol. Biol.* (2003) **328**, 1047–1060

JMB

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Energetics of Lesion Recognition by a DNA Repair Protein: Thermodynamic Characterization of Formamidopyrimidine-glycosylase (Fpg) Interactions with Damaged DNA Duplexes

Conceição A. S. A. Minetti[1]†, David P. Remeta[1]†, Dmitry O. Zharkov[2] G. Eric Plum[1], Francis Johnson[2], Arthur P. Grollman[2] and Kenneth J. Breslauer[1]

[1]*Department of Chemistry and Chemical Biology, Rutgers University, Wright Chemistry Bldg, 610 Taylor Road Rm 0156, Piscataway, NJ 08854-8087, USA*

[2]*Laboratory of Chemical Biology, Department of Pharmaceutical Sciences SUNY Stony Brook, Stony Brook, NY 11794, USA*

As part of an overall effort to map the energetic landscape of the base excision repair pathway, we report the first thermodynamic characterization of repair enzyme binding to lesion-containing duplexes. Isothermal titration calorimetry (ITC) in conjunction with spectroscopic measurements and protease protection assays have been employed to characterize the binding of *Escherichia coli* formamidopyrimidine-glycosylase (Fpg), a bifunctional repair enzyme, to a series of 13-mer DNA duplexes. To resolve energetically the binding and the catalytic events, several of these duplexes are constructed with non-hydrolyzable lesion analogs that mimic the natural 8-oxo-dG substrate and the abasic-like intermediates. Specifically, one of the duplexes contains a central, non-hydrolyzable, tetrahydrofuran (THF) abasic site analog, while another duplex contains a central, carbocyclic substrate analog (carba-8-oxo-dG). ITC-binding studies conducted between 5.0 °C and 15.0 °C reveal that Fpg association with the THF-containing duplex is characterized by binding free energies that are relatively invariant to temperature ($\Delta G \sim -9.5$ kcal mol^{-1}), in contrast to both the reaction enthalpy and entropy that are strongly temperature-dependent. Complex formation between Fpg and the THF-containing duplex at 15 °C exhibits an unfavorable association enthalpy ($\Delta H = +7.5$ kcal mol^{-1}) that is compensated by a favorable association entropy ($T\Delta S = +17.0$ kcal mol^{-1}). The entropic nature of the binding interaction, coupled with the large negative heat capacity ($\Delta C_p = -0.67$ kcal deg^{-1} mol^{-1}), is consistent with Fpg complexation to the THF-containing duplex involving significant burial of non-polar surface areas. By contrast, under the high ionic strength buffer conditions employed herein (200 mM NaCl), no appreciable Fpg affinity for the carba-8-oxo-dG substrate analog is detected. Our results suggest that initial Fpg recognition of a damaged DNA site is predominantly electrostatic in nature, and does not involve large contact interfaces. Subsequent base excision presumably facilitates accommodation of the resulting lesion site into the binding pocket, as the enzyme interaction with the THF-containing duplex is characterized by high affinity and a large negative heat capacity change. Our data are consistent with a pathway in

† C.A.S.A.M. and D.P.R. contributed equally to this study.

Present address: D. O. Zharkov, Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of Russian Academy of Sciences, Novosibirsk 630090, Russian Federation.

Abbreviations used: AP, abasic site; Fpg, formamidopyrimidine glycosylase; hOGG1, human oxoguanine glycosylase 1; ITC, isothermal titration calorimetry; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; THF, tetrahydrofuran; UDG, uracil-DNA glycosylase.

which Fpg glycosylase activity renders the base excision product a preferred ligand relative to the natural substrate, thereby ensuring the fidelity of removing highly reactive and potentially mutagenic abasic-like intermediates through catalytic elimination reactions.

Keywords: protein–DNA interactions; energetics; Fpg; repair enzyme; abasic site

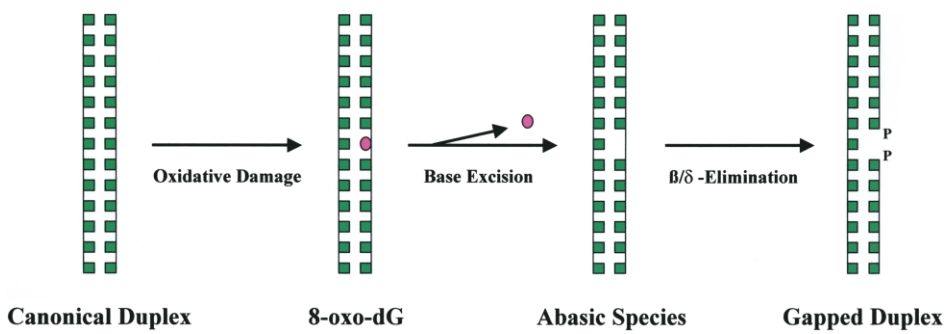
Introduction

The abasic site is a ubiquitous and highly mutagenic DNA lesion that can be generated by spontaneous depurination or by the excision repair of damaged bases. Abasic sites (APs) or their derivatives are products of monofunctional DNA glycosylases, reaction intermediates of DNA glycosylases/AP lyases, and substrates for repair enzymes that remove these lesions either by hydrolysis or β-elimination (for a review refer to David & Williams).[1] Non-hydrolyzable analogs of abasic sites represent reasonable models for enzyme recognition/binding studies. In fact, several non-hydrolyzable analogs have been reported to exhibit high affinity for repair enzymes.[2–4] Studies on enzyme binding to duplexes containing such abasic site analogs therefore constitute a logical starting point for mapping the thermodynamic landscape of the base excision repair process.

Formamidopyrimidine DNA glycosylase (Fpg) protein, which is also known as Fapy-DNA glycosylase, 8-oxoguanine-DNA glycosylase, and MutM, is an integral part of the base excision repair system in *Escherichia coli*. Fpg initiates repair of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxo-dG), a DNA lesion generated by oxygen free radicals.[5] This bifunctional repair enzyme possesses both DNA glycosylase and AP lyase activities.[6] Scheme 1 depicts DNA states associated with Fpg glycosylase and lyase recognition and repair activities. The reaction cascade includes an initial encounter of Fpg with either a damaged or canonical site within the target duplex domain, followed by excision of the damaged base *via* the enzyme's glycosylase activity, and release of the gapped duplex product for subsequent repair by other enzymes.

As an initial system for thermodynamic characterization, we have selected the association of Fpg with oligonucleotides containing the tetrahydrofuran (THF) moiety, a non-cleavable abasic site analog. This analog lacks the C-1 hydroxyl group of the 2-deoxyribose[2] and is well suited for recognition studies, since it binds but is not cleaved by Fpg.[3,5] Abasic site analogs resemble both the natural abasic site (a known substrate for Fpg), and the transient Fpg-DNA imino intermediate between Pro1 of the enzyme and C1′ of the damaged nucleotide.[7] A concerted mechanism of Schiff base formation followed by lyase activity (*via* β and δ-elimination) results in formation of a gapped DNA product. By incorporating abasic site analogs within a duplex substrate, we can eliminate the catalytic event that occurs subsequent to abasic site recognition. This feature allows us to isolate and thermodynamically characterize the binding/recognition event between a repair enzyme and the abasic site.

High-resolution crystal structures of Fpg from *Thermus thermophilus*[8] and Fpg associated with analogs of damaged DNA ligands from *Lactobacillus lactis*[9] or *Bacillus stearothermophilus*[10] have furnished valuable information regarding the free enzyme *versus* the DNA-bound complex. Sodium borohydride reduction of an obligatory Schiff base intermediate produces a covalent complex between *E. coli* Fpg and a DNA substrate (with the base lesion already excised). Elucidation of the resultant structure has provided significant insight into specific binding/recognition features of the *E. coli* Fpg·abasic site complex.[11] Such structural studies of the Fpg·DNA complex provide us with a microscopic context within which to interpret our macroscopic data, thereby further justifying our selection of *E. coli* Fpg as the repair enzyme for thermodynamic analysis.



Scheme 1. Putative substrates/intermediates/products of Fpg glycosylase/lyase activities.

As an initial step in defining the thermodynamic profile of glycosylase-mediated DNA repair, we have employed isothermal titration calorimetry (ITC) to provide the first complete thermodynamic characterization of repair enzyme association to a lesion-containing DNA duplex. As a model for both a product of DNA glycosylase activity and a substrate for AP lyase activity, the THF abasic site complexed with Fpg represents a suitable mimic of a critical intermediate in the repair process. Consequently, it is an ideal target for our initial thermodynamic mapping of the energetic landscape associated with the cascade of events along the "reaction coordinate" of Fpg base excision repair.

Results and Discussion

Experimental design considerations

Footprint experiments[3] in conjunction with crystallographic analysis[11] reveal that Fpg·DNA interactions establish contacts with DNA ligands, which span no more than nine base-pairs. Consequently, the use of 13-mer oligonucleotide duplexes as targets for the binding studies should preclude multiple site binding, yet exhibit the requisite duplex thermal stability over the temperature range and solution conditions employed in the energetic studies. Ensuring the integrity of the folded initial states represents another issue that must be assessed in studies dealing with the energetics of protein–ligand interactions.[12] In terms of the DNA duplexes studied herein, analysis of the concentration-dependent UV melting profiles reveals that the 13-mer duplexes are fully associated under the buffer conditions employed over the temperature (i.e. 5.0–15.0 °C) and concentration (i.e. 10–20 μM) ranges used in the ITC experiments. Inspection of the UV melting profiles acquired at micromolar concentrations indicates no significant changes in the association state of the DNA duplexes at temperatures below 25 °C, a value well above the upper temperature limit (15 °C) of the ITC measurements (results not shown). The thermal stability of *E. coli* Fpg has been assessed by monitoring its temperature-dependent UV absorbance. The resultant data reveal that the free protein undergoes irreversible thermal unfolding with a t_m of ~65 °C (data not shown). It is therefore apparent that both the protein and DNA duplexes are fully folded/associated over the temperature range of our binding studies.

Under the high ionic strength conditions used in this study (232 mM total Na^+), Fpg operates primarily in the distributive mode (J. Tchou & A.P.G., unpublished studies on plamid DNA), exhibiting negligible binding to canonical DNA duplexes.[3] Consequently, we can thermodynamically characterize specific protein–DNA interactions in the absence of non-specific binding. Referring to the cascade of DNA states depicted in Scheme 1, the abasic-containing duplex resembles the intermediates formed by the enzyme's DNA glycosylase activity, while the gapped duplex represents the product of the enzyme's AP lyase activity. We have designed and synthesized several stable, non-hydrolyzable structural mimics of the abasic site lesion (a THF derivative and a carbocyclic derivative), and the gapped product (a 13-mer duplex with a central gap). These structural analogs of natural Fpg ligands allow us to investigate protein–DNA binding/recognition in the absence of catalysis, thereby furnishing insight into the energetic landscape of the enzyme's mechanism of action.

Fpg·DNA interactions are not accompanied by significant spectroscopically detected alterations in the global conformation of the components in the complex

Circular dichroism (CD) spectra of the free oligonucleotide duplexes reveal a characteristic *B*-DNA conformation with relatively minor quantitative differences. Inspection of the CD spectra presented in Figure 1(a) reveals that, consistent with our previous observations,[13] the intensity of the positive band centered between 260 nm and 290 nm is reduced in the THF-containing *versus* the canonical duplex, as one would expect for the removal of a chromophore. Figure 1(b) compares the CD spectrum of free Fpg (assessed over the range of 200–250 nm) with that of the difference spectra calculated for the protein in the presence of the canonical or THF–DNA duplexes. The difference "binding" spectra are obtained by subtracting the reference spectra of the respective free DNA duplexes from the spectra of the corresponding protein–DNA "complexes". The CD spectra of Fpg and a 1:1 Fpg·DNA mixture reveal that the overall native-like Fpg protein secondary structure remains unchanged in the presence of DNA. The near superimposition of these spectra indicates that little or no significant global structural reorganization occurs upon complexation of Fpg with the THF-containing duplex, despite independent calorimetric, protease protection, and gel shift evidence for Fpg·THF–DNA binding presented below. Our spectroscopic-based evidence that there is no significant global structural reorganization upon complexation of Fpg with the THF-containing duplex is consistent with existing structural data. However, as noted below, structural studies reveal some binding-induced local loop reorganization.

Crystallographic analysis of the free *Tth* Fpg protein[8] in comparison with a number of Fpg·DNA complexes[9–11] provides no indication for an induced-fit mechanism. These structural comparisons suggest that DNA binding does not alter the global conformation of the Fpg protein. This result contrasts with a number of other DNA-binding proteins, for which significant disorder–order transitions have been identified.[14,15] However, despite

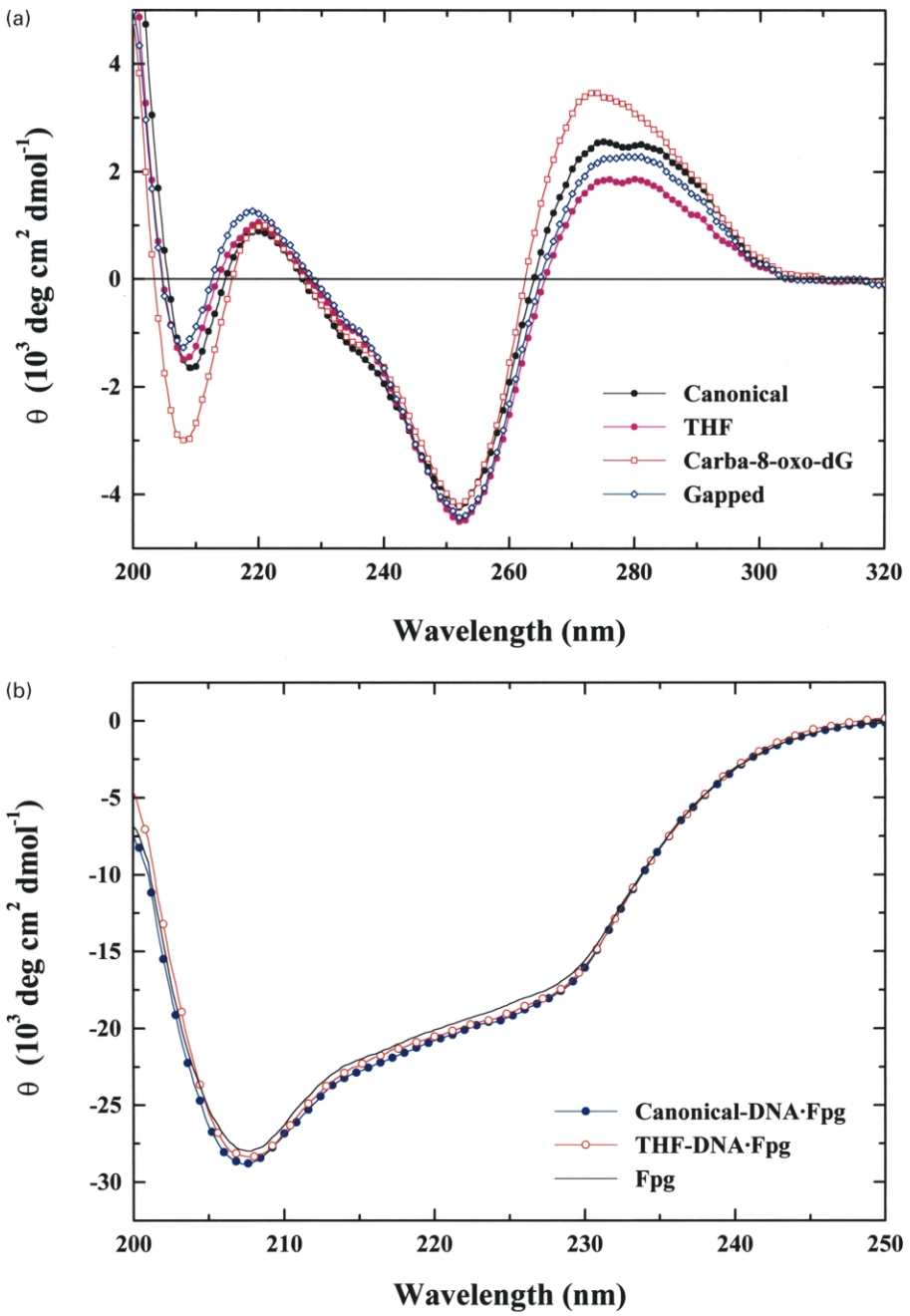


Figure 1. CD spectra of a series of 13-mer DNA duplexes and the resultant complexes formed with Fpg at 5.0 °C. (a) Comparison of CD spectra for the lesion-containing 13-mer DNA duplexes and their canonical counterpart. (b) Comparison of the CD spectra of free Fpg with that of the difference spectra calculated for the protein in the presence of the canonical or THF–DNA duplexes. The difference binding spectra are obtained by subtracting the reference spectra of the free DNA duplexes from the spectra of the corresponding Fpg·DNA complexes.

the lack of DNA binding-induced alterations in global protein structure, all of the Fpg·DNA complexes (i.e. *E. coli*, *L. lactis*, and *B. stearothermophilus*) characterized to date[9–11] furnish direct or indirect evidence for local, binding-induced disorder of a short loop (residues 217–224 in *E. coli*) that is ordered in the free *Tth* Fpg.[8] This local loop reorganization has been independently confirmed by crystallographic studies on endonuclease VIII, another glycosylase member of this family of repair enzymes (G. Shoham, unpublished results). In the aggregate, the structural and spectroscopic evidence is consistent with the absence of any significant DNA binding-induced global protein folding.

Protease protection assays: abasic-containing DNA duplexes protect Fpg against protease digestion

Electrophoretic studies of Fpg protease resistance have been employed to monitor the shielding

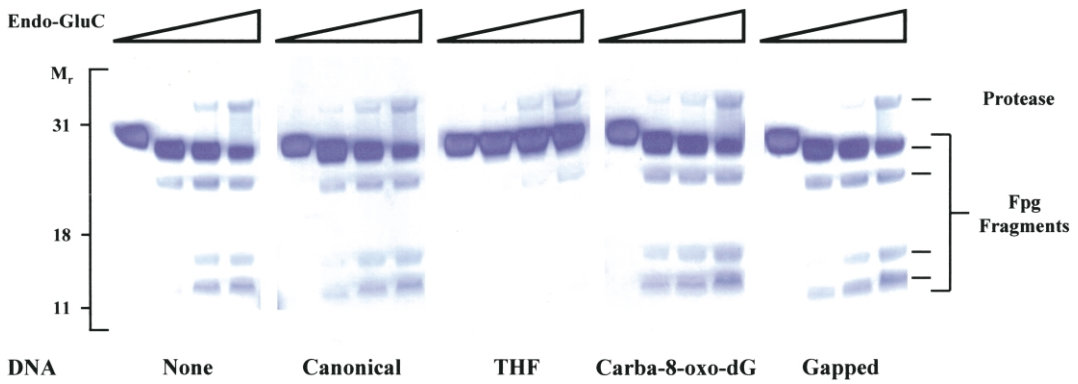


Figure 2. Protease protection assays of free Fpg and the resultant complexes formed between Fpg and selected 13-mer DNA duplexes (i.e. canonical, THF, carba-8-oxo-dG, and gapped). Increasing endoproteinase Glu-C concentrations have been added to the reaction mixtures (at a w/w ratio of 0, 0.02, 0.1, and 0.2 protease: Fpg, respectively) as noted by the concentration gradients at the top of the gels. The binding reactions (at a ratio of 2:1 DNA:Fpg) have been conducted by incubating the complexes for 30 minutes, followed by addition of the indicated concentrations of protease, and subsequent termination after 30 minutes by denaturation with LDS-sample buffer and heating to 95 °C for three minutes prior to loading the gel. The protease band and the resultant Fpg digestion fragments are designated by the labeled bars on the right side of the gels.

effects of Fpg complexation with DNA. A protease protection experiment aimed at obtaining a "protein footprint" for Fpg·DNA interactions has been performed by endoproteinase Glu-C digestion of free Fpg and Fpg in the presence of the DNA duplexes studied herein (Figure 2). When low protease concentrations are employed, Fpg exhibits a single cleavage site (yielding a ~25 kDa fragment), whether in the absence or presence of DNA, presumably located within the C-terminal domain as verified in *E. coli* Endo VIII (D.O.Z., unpublished results). Increasing protease concentrations result in the appearance of additional bands in the Fpg digestion profile at the expense of the truncated larger fragment, the latter decreasing proportionally in density. Pre-incubation of Fpg with the THF-containing duplex, followed by subsequent protease treatment yields a characteristic digestion pattern, consistent with binding-induced protection of Fpg. The results are comparable irrespective of the abasic site analog employed (i.e. THF or α-carba-abasic, data not shown). In contrast, no Fpg protection is observed in the presence of the canonical duplex, a control that is consistent with the lack of Fpg binding to the lesion-free duplex. We also observe no Fpg protection in the presence of the carba-8-oxo-dG-containing duplex, nor the corresponding 13-mer gapped DNA duplex. These results suggest that unlike the abasic site containing analogs, the remaining duplexes studied herein exhibit a distinct binding mode that involves neither large interfaces nor the burial of specific endo Glu-C protease sites. The results of our protection studies are consistent with selective recognition/binding of the THF-containing DNA duplex by the Fpg repair protein and set the stage for our calorimetric studies of Fpg binding to this duplex.

Calorimetric characterization of Fpg-binding affinity for a THF-containing duplex

We have employed ITC to evaluate the binding affinity of Fpg for a 13 base-pair DNA duplex containing a central abasic (THF) site. The high ionic strength buffer used in the ITC experiments serves a dual purpose, namely to permit assessment of the binding energetics under conditions whereby the lesion-containing duplex is fully associated, and to minimize non-specific protein–DNA association interactions.[3] A representative binding isotherm acquired at 5.0 °C for the interaction of the THF-containing DNA duplex with Fpg is presented in Figure 3. The upper panel displays the heat detected per injection of Fpg into the THF-containing DNA duplex, while the lower panel presents the integrated data and the non-linear least-squares fit to a single site-binding model. The resultant ITC profile is characterized by a stoichiometric ratio of one protein molecule per DNA duplex and an overall binding affinity (K_a) of $1.95 \times 10^7\ M^{-1}$. The corresponding binding free energy for the Fpg·DNA association reaction is calculated from the standard thermodynamic relation:

$$\Delta G^\circ = -RT \ln K_a \qquad (1)$$

where ΔG° represents the Gibbs free energy, K_a is the binding affinity, T is the temperature in Kelvin, and R is the universal gas constant ($1.987\ cal\ K^{-1}\ mol^{-1}$). Employing the calorimetrically measured binding affinity, we calculate a binding free energy of $-9.3\ kcal\ mol^{-1}$ at 5.0 °C for the interaction of Fpg with the abasic THF-containing DNA duplex.

The significant free energy of binding for Fpg and THF–DNA contrasts with the negligible binding affinity detected by ITC for the interaction

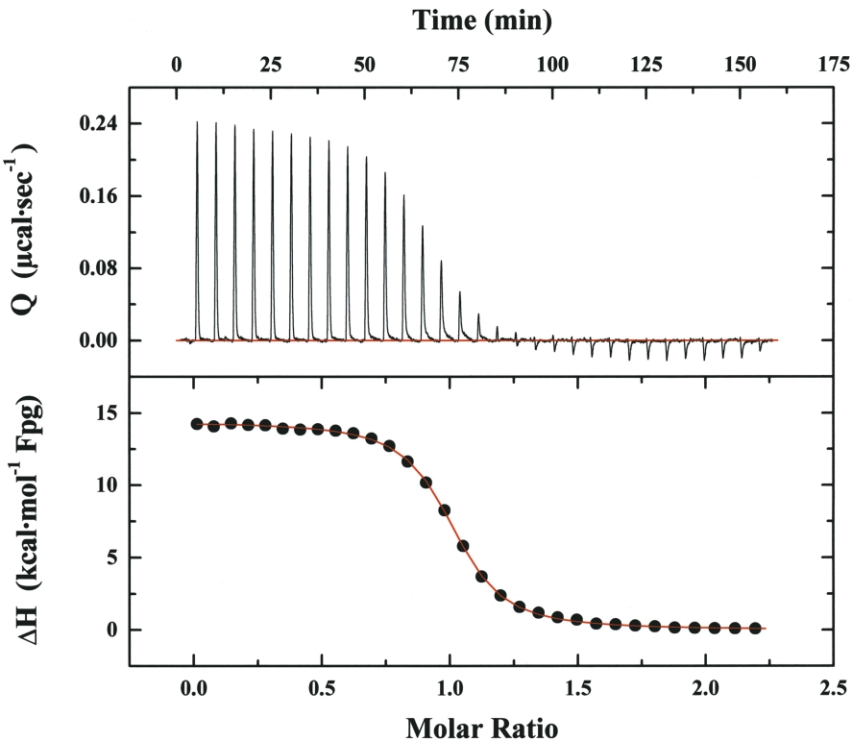


Figure 3. ITC binding isotherm for the association of a 13-mer DNA duplex containing a central abasic (THF) site with Fpg at 5.0 °C in 20 mM sodium phosphate, 200 mM NaCl 1 mM DTT at pH 7.0. Upper, Total heat absorbed upon injecting 7.5 μl aliquots of Fpg (75 μM protein) into a 1.4246 ml reaction cell containing THF–DNA (10 μM duplex). Lower, Resultant-binding isotherm (circles) obtained by integrating the peak areas of each injection. The continuous line represents the non-linear least-squares fit of the affinity (K_a), enthalpy (ΔH), and stoichiometry (n) to a single-site-binding model.

between Fpg and the corresponding canonical DNA duplex under identical temperature and buffer conditions. This observation suggests that the high ionic strength effectively screens out non-specific-binding interactions, which apparently represent the primary interactions between the protein and canonical DNA. This finding corroborates gel shift assays performed at NaCl concentrations greater than 150 mM.[3] As an added precaution to ensure that our enthalpy-based binding assay does not fail to detect entropically driven Fpg·DNA complex formation, we also performed a competitive titration experiment in which we titrated Fpg into an equimolar mixture containing both the THF-containing and canonical DNA duplexes. In this ITC competition experiment, we obtained nearly identical values for K_a, ΔH, and stoichiometry as observed in the absence of a competing ligand. Taken together, these results are consistent with the THF-containing duplex binding to Fpg with far greater affinity than the canonical DNA sequence, particularly under the high ionic strength conditions employed herein.

The enthalpic and entropic driving forces of Fpg·THF–DNA complexation

One of the unique advantages of ITC is that a single, well-designed experiment furnishes the requisite energetic data to derive a complete thermodynamic profile for Fpg·DNA using the standard thermodynamic relation:

$$\Delta G = \Delta H - T\Delta S \qquad (2)$$

where $\Delta G°$ represents the Gibbs free energy, $\Delta H°$ is the binding enthalpy, and $\Delta S°$ the resultant association entropy. Table 1 presents the thermodynamic-binding parameters determined for Fpg association with an abasic THF-containing DNA duplex at several different temperatures. Dissection of the binding free energy ($\Delta G = -9.3$ kcal mol^{-1}) determined at 5.0 °C into its enthalpic ($\Delta H = +14.2$ kcal mol^{-1}) and entropic ($T\Delta S = +23.5$ kcal mol^{-1}) components reveals that complexation of Fpg with the THF-containing duplex is exclusively an entropy-driven process that is characterized by a strongly unfavorable binding enthalpy, which remains unfavorable at temperatures below 25 °C. This thermodynamic behavior is consistent with significant binding-induced desolvation of protein and DNA surfaces upon complex formation.[12,16,17] In order to dissect the apparent enthalpic component into its intrinsic association enthalpy and coupled equilibria (i.e. linked protonation/deprotonation), calorimetric measurements have also been performed in buffers

Table 1. Thermodynamic binding profiles for association of Fpg with the THF–DNA duplex

Temperature (°C)	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$T\Delta S°$ (kcal mol^{-1})
5.0	−9.3	+14.2	+23.5
10.0	−9.4	+10.9	+20.3
15.0	−9.5	+7.5	+17.0
25.0[a]	−9.7	+0.8	+10.5

The uncertainties in the reported values of $\Delta G°$, $\Delta H°$, and $T\Delta S°$ are within ±0.1 kcal mol^{-1}.

[a] Thermodynamic-binding parameters extrapolated to 25.0 °C based on a ΔC_p of −0.67 kcal mol^{-1} K^{-1}.

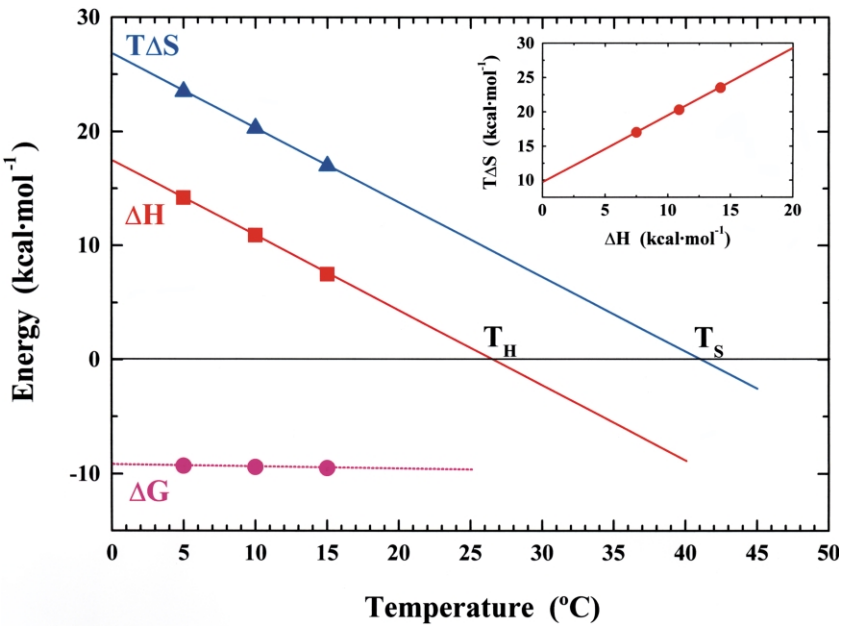


Figure 4. Temperature-dependence of the thermodynamic parameters derived for the association of THF-containing DNA duplex to Fpg. The linear fits of ΔH and $T\Delta S$ are extrapolated to zero where T_H and T_S correspond to the temperatures at which ΔH and $T\Delta S$ change sign, respectively. Inset: enthalpy *versus* entropy compensation for the THF–DNA·Fpg interaction.

of distinct ionization enthalpies (results not shown). The nearly identical association enthalpies observed for Fpg binding to the THF-containing duplex in different buffer systems reveal that the interaction does not involve linked protonation effects, and therefore the empirically derived binding heats reflect the intrinsic Fpg·THF–DNA-binding enthalpy.

Fpg·THF–DNA binding free energies are temperature-invariant

Further inspection of the data in Table 1 reveals that the binding free energy is relatively invariant over the temperature range of 5.0–15.0 °C. Despite comparable binding free energies over this temperature range, we observe substantial and compensating temperature-dependent changes in both the enthalpic and entropic terms. Figure 4 depicts a temperature extrapolation of the enthalpic and entropic contributions to the overall binding free energy. Note that the binding enthalpy remains endothermic throughout the temperature range studied, and changes sign at ~26 °C, designated the T_H. Similarly, the binding entropy changes sign at ~40.0 °C, the corresponding T_S. Analogous to the dissolution of non-polar model compounds in aqueous media and burial of apolar residues in protein folding, it has been proposed that the enthalpy of interactions for which the hydrophobic effect plays a major role approaches zero at about 25 °C (Privalov *et al.* and references contained therein).[12,17] Taken together, our enthalpy and entropy data, as well as their temperature dependences, are consistent with Fpg·DNA interactions being dominated by hydrophobic contacts. As discussed in the following section, the implication that Fpg·DNA association involves significant hydrophobic interactions is consistent with the overall magnitude of the binding affinity and large negative heat capacity observed in the presence of high ionic strength.

Association of Fpg with the THF-containing duplex is accompanied by a relatively large negative heat capacity change

Inspection of the data in Table 1 and the slope in Figure 4 reveals that THF–DNA binding to Fpg is accompanied by a negative change in heat capacity (ΔC_p) equal to -0.67 kcal deg^{-1} mol^{-1}. Sequence-specific protein–DNA interactions generally are high-affinity binding events characterized by substantial negative heat capacity changes, presumably reflecting binding-induced dehydration of non-polar groups.[12,15,18–20] Published values of ΔC_p for specific protein–DNA interactions generally range from -0.58 kcal deg^{-1} mol^{-1} to -0.95 kcal deg^{-1} mol^{-1}. Our empirically derived ΔC_p value of -0.67 kcal deg^{-1} mol^{-1} for interaction of the abasic-containing DNA duplex with Fpg falls within this range. Such changes in heat capacity are consistent with significant burial of apolar surfaces upon binding, which perforce involves binding-induced desolvation of the protein–DNA interface, a phenomenon consistent with entropically driven binding events.

We can use our experimentally determined heat capacity data to extrapolate the thermodynamic driving forces for Fpg–DNA complexation at any desired temperature. Assuming $d\Delta C_p/dT = 0$, and using our ΔC_p value of -0.67 kcal deg^{-1} mol^{-1} for the *E. coli* Fpg·THF–DNA complex, we calculate that the process is driven by favorable entropic contributions ($T\Delta S = +10.5$ kcal mol^{-1}), with a lower enthalpic inhibition ($\Delta H = +0.8$ kcal mol^{-1}) at 25 °C (Table 1). The thermodynamic properties of Fpg binding for which there is no sequence-specific requirement[3] resemble those reported for a number of site-specific DNA-binding

proteins,[12,21] reflecting primarily the removal of non-polar surfaces from water in the association process. It is interesting to note, however, that the thermodynamic binding parameters for Fpg·THF–DNA complexation at 37 °C are characteristic of an enthalpy driven ($\Delta H \sim -7.0$ kcal mol^{-1}) interaction with favorable entropic contributions ($T\Delta S \sim +2.5$ kcal mol^{-1}). The favorable enthalpic contribution observed at higher temperatures clearly illustrates how the relative compensation by desolvation of apolar residues and release of cations (which drive thermodynamically unfavorable processes at lower temperatures) might not play a major role in yielding complementary recognition surfaces at higher temperatures. This observation may also reflect the reduced hydration enthalpies of apolar groups at higher temperatures relative to the influence of van der Waals interactions.

Comparison of empirical and calculated ΔC_p changes derived from binding-induced burial of solvent-accessible surface areas (SASA)

While the heat capacity change upon binding reflects the re-distribution of available energetic states, there have been attempts to empirically relate the ΔC_p parameter with specific features of macromolecular structure, as determined from X-ray crystallographic analysis. Spolar & Record[15] have proposed a relationship to estimate the heat capacity change associated with macromolecular associations and reorganizations; namely, $\Delta C_p = [0.32(\pm 0.04)\Delta A_{NP} - 0.14(\pm 0.04)\Delta A_P]$ cal mol^{-1} K^{-1}, where A_{NP} and A_P are the binding-induced changes in non-polar and polar solvent-accessible surface areas expressed in units of Å^2, respectively. The total surface area buried within the cross-linked, borohydride-reduced Schiff base intermediate of the *E. coli* Fpg·8-oxo-dG DNA complex is 2512 Å^2.[11] The surface area buried by binding of *L. lactis* Fpg to an abasic site, which may more closely parallel our non-covalent complex, is 2000 Å^2.[9] Employing the NACCESS software program to derive appropriate parameters for the buried polar and non-polar surface areas in these crystal structures, we calculate a ΔC_p of -0.25 kcal mol^{-1} deg^{-1} for the *E. coli* Fpg·8-oxo-dG DNA complex and a ΔC_p of -0.22 kcal mol^{-1} deg^{-1} for the *L. lactis* Fpg·abasic DNA complex. Although these calculated values are of the same sign as our experimentally derived ΔC_p of -0.67 kcal deg^{-1} mol^{-1} for the *E. coli* Fpg·THF–DNA complex, both are sufficiently lower in terms of absolute magnitude to warrant further comment. In protein–ligand-binding studies (including protein–DNA complexation), a common finding is that the experimental ΔC_p values exceed those calculated exclusively from structural considerations.[16,20,22,23] This apparent disparity has been associated with coupled events, including protein unfolding/folding mechanisms[14] and/or binding-induced DNA unstacking,[24] both of which will contribute to the heat capacity term. Since our results are consistent with the observed absence of significant global rearrangements in protein structure, we suggest that the higher absolute ΔC_p value (compared with that calculated from the net burial of apolar surface area) may result from coupled, binding-induced equilibria that are not detected in CD spectra. Fpg binding also could induce enthalpically unfavorable distortions in the DNA duplex as suggested for other protein–DNA systems by the Jen-Jacobson group.[25] Indeed, significant base unstacking and kinking have been reported from structural analysis of Fpg·damaged DNA complexes.[9–11] Such protein-induced DNA distortions are enthalpically unfavorable,[25–27] and may contribute, at least in part, to the endothermic binding that we observe below 25 °C. Although the identity of the lesion as well as the sequence context of the DNA co-crystallized with *E. coli* Fpg differs from that used in our solution study, it is conceivable that base unstacking may contribute an additional unfavorable enthalpy to the binding free energy. Binding induced-DNA unstacking has been reported to exhibit a negative ΔC_p as observed for SSB protein–DNA interactions.[24] Therefore, the unfavorable enthalpic contributions due to base unstacking may result in both an overestimate of ΔC_p and an endothermic contribution to the overall binding enthalpy. Furthermore, as emphasized by Sturtevant,[16] the binding-induced changes in heat capacity may reflect contributions from a reduction in the soft vibrational modes available to the protein and the DNA in the complexed state. These additional binding-induced considerations are either not fully or formally incorporated into existing algorithms for calculating heat capacity changes. The structural and thermodynamic characterizations of the *E. coli* Fpg·THF–DNA complex suggest that a model in which heat capacity changes are parameterized solely in terms of buried surface area does not adequately describe the phenomena underlying the experimentally determined ΔC_p. The entropic contribution is presumably dominated by displacement/reorganization of solvent and displacement of counterions from the two interacting macromolecular surfaces. The relative contributions of each of these effects coupled with conformational reorganizations and alterations of vibrational modes[16] of the macromolecules cannot be resolved from the available data. Nonetheless, it is conceivable that conformational changes (albeit silent in CD spectra and crystallographic analysis) involving the removal of additional non-polar surface of the protein and DNA from water may contribute to the large negative ΔC_p measured for the Fpg·THF–DNA complex.

Fpg-binding studies employing other non-hydrolyzable substrate analogs

Mapping the energetic landscape for the cascade of events associated with the catalytic activity of an

enzyme represents a challenging task. Thermodynamic characterizations of enzyme–substrate/intermediate/product interactions can be complicated by binding and catalytic events occurring concurrently, thereby precluding energetic resolution of individual steps. To address this challenge, we have designed and synthesized structural analogs that bind but are not cleaved by Fpg. The THF abasic site described above represents one such structural analog. In the sections that follow, we describe the characterization of other classes of structural analogs that mimic the 8-oxo-dG substrate, the abasic imino intermediates, and the gapped duplex product.

The carbocyclic derivative of 8-oxo-dG exhibits no measurable affinity for Fpg at high ionic strength

Carbocyclic nucleoside analogs that differ in composition from their parent compounds merely by substitution of CH_2 for O4′ have been employed as tools for elucidating the mechanisms of recognition and catalysis by repair enzymes.[4,7,28] The carba-8-oxo-dG analog resists cleavage by Fpg, since the lack of O4′ prevents formation of the aminal intermediate and subsequent rearrangement into a Schiff base intermediate.[7] Although extensive structural and biochemical studies have aimed at elucidating the mechanisms involved in the initial recognition events by repair enzymes, these processes remain unresolved. Our ITC studies reveal that, unlike the THF-containing duplex, we do not detect an appreciable Fpg affinity for the carba-8-oxo-dG lesion. This result suggests that under the ionic strength conditions employed herein, initial recognition of the 8-oxo-dG lesion by Fpg does not involve a high-affinity binding interaction. This unexpected finding implies that initial recognition of the damaged base may not involve appreciable surface burial or water release, and might be driven predominantly by coulombic electrostatic interactions, which are screened out under "high" salt conditions. Significantly, Fpg is active under these ionic strength conditions as confirmed by HPLC analysis of the Fpg·8-oxo-dG reaction products (results not shown), albeit exhibiting maximal efficiency at $[Na^+] \sim 100$ mM as reported previously.[29] Such salt-dependent behavior is well established for repair enzymes[30,31] and reflects a switch from the processive to the distributive mode of action at certain salt concentrations that are characteristic for each enzyme.

The carbocyclic abasic site binds to Fpg with high affinity

We have evaluated the Fpg binding properties of a carbocyclic derivative of the natural abasic site to assess whether carbocyclic modification (an O4′ to CH_2 replacement in the deoxyribose ring) impacts Fpg binding/recognition independent of the lesion itself. Previous studies have demonstrated that substitution of O4′ in the THF moiety yields a carba-abasic analog that actually exhibits higher affinity for either *L. lactis* or *E. coli* Fpg when compared with the THF-containing duplex.[4] Initial studies employing carbocyclic derivatives of natural abasic site stereoisomers reveal that the resultant lesion-containing duplexes bind to Fpg with high affinity and exhibit similar protease protection profiles to the THF analog (results not shown). ITC measurements of the interaction of Fpg with the α-carba-abasic-containing duplex reveal the characteristic entropically driven association at low temperatures (5 °C). Thermodynamic binding parameters derived from analysis of the resultant profiles are comparable to the THF analog. Significantly, temperature-dependent studies indicate that the ΔC_p for the interaction with duplexes containing either THF or the α-carba-abasic site do not differ significantly (C.A.S.A.M. and co-workers, unpublished results). These findings support the notion that Fpg interactions with abasic site-containing duplexes (characterized herein as reflecting the burial of large apolar surface areas) are not precluded by an O4′ to CH_2 replacement in the deoxyribose ring. Based on these results, we suggest that the lack of Fpg binding to the carbocyclic 8-oxo-dG derivative does not actually reflect the influence of the carbocyclic modification, but rather the intrinsically lower affinity of Fpg for the carba-8-oxo-dG (and conceivably the 8-oxo-dG) lesion-containing duplex compared with the abasic-containing duplex. The differential Fpg affinity observed for the glycosylase-generated abasic intermediate relative to the nascent 8-oxo-dG lesion substrate may have important functional implications.[32] We may postulate that following a relatively weak, electrostatically dominated initial recognition by Fpg of the 8-oxo-dG lesion-containing duplex, the glycosylase activity of the repair enzyme (base excision) generates a more favorable abasic-like Fpg substrate ligand. Confirmation of this hypothesis awaits elucidation of the interactions of catalytically inactivated mutant Fpg forms with the natural 8-oxo-dG substrate-containing DNA. Such site-directed mutated enzymes will allow us to use the natural 8-oxo-dG-containing substrates to analyze Fpg·DNA binding energetics in the absence of subsequent catalytic events.

Implications for the Fpg·DNA energy landscape: lesion recognition, base excision/abasic site creation, and product release

Scheme 1 illustrates the DNA states associated with Fpg catalytic activity, which includes an initial non-specific encounter of Fpg with either a damaged or canonical site within the duplex, a multistep base recognition process, base excision, followed by β and δ-elimination reactions that release the damaged base and gapped DNA products for repair by other enzymes. In terms of initial recognition of the 8-oxo-dG lesion by Fpg, there must be some level of selective interaction

with this lesion by the repair enzyme, despite our calorimetric, protease protection, and gel shift data, which collectively indicate no detectable binding under the solution conditions employed herein. Since our high salt conditions favor the distributive reaction profile, perhaps the initial interaction of Fpg with the 8-oxo-dG containing duplex DNA does not involve an extensive binding interface, but rather is governed by "soft" coulombic electrostatic interactions, which are screened out in our high salt assay. The possibility of such soft, non-specific interactions occurring as an initial step in damaged base recognition, followed by more specific H-bonding interactions, is supported by *ab initio* quantum-mechanical calculations,[33] whereby the potential around C_8-N_7 provides a highly discriminative power to distinguish between canonical *versus* damaged bases. Additional insight into Fpg substrate recognition arises from time-resolved fluorescence studies of the *E. coli* Fpg catalytic process.[34] An initial phase has been ascribed to the encounter with both 8-oxo-dG-containing as well as the corresponding undamaged duplexes, for which rate constants have been derived. The calculated K_D values (i.e. $\sim$1.3 μM and 12 μM for 8-oxo-dG-DNA and undamaged DNA, respectively) may not be experimentally accessible under conditions of high ionic strength. Assuming comparable K_D values for the carba-8-oxo-dG derivative, the resultant Fpg·DNA-binding interactions may be screened out at high salt concentrations, thereby rendering calorimetric measurements impractical. Recall that at high salt we observe strong Fpg binding to the abasic-containing duplexes and no detectable binding to the corresponding carba-8-oxo-dG-containing duplex. This differential may well reflect the electrostatic nature of the initial lesion recognition event, whereby relatively weak discrimination association of Fpg with damaged base sites precedes glycosylase-mediated base excision that creates a preferred abasic-like duplex domain and conceivably a better substrate for further catalysis by Fpg. The dramatic difference in binding free energies observed in the ground state (prior to and following base excision) may in fact represent a driving force favoring the progress of the reaction towards completion.

Further support for our interpretation of the origins of the enhanced affinity of Fpg for the abasic site-containing duplex *versus* the damaged base-containing duplex arises from the results of several published studies. THF-containing duplexes have been reported to inhibit the catalytic activity of Fpg,[4] consistent with a higher affinity for Fpg of the THF-containing duplex relative to the natural Fpg substrate. The origins of this disparity, particularly for a compound that resembles the natural damaged substrate (i.e. carba-8-oxo-dG), may be related to a significantly lower surface contact area when the damaged base is present (at least as far as ground state binding is concerned). There is evidence to support such a notion in that the proposed hOGG1 substrate pocket for 8-oxo-dG[35] as probed with a mutant hOGG1, is significantly smaller than the pocket for an abasic THF site.[36] Although a smaller pocket may allow greater precision for catalysis by restricting the lesion into the proper orientation, the larger binding pocket observed for THF in hOGG1 is consistent with the burial of a more extensive surface area, as well as the favorable entropy and high ΔC_p we measure for the interaction of Fpg with the THF-containing duplex. Furthermore, the estimated binding free energy for the UDG-mismatched base complex is significantly less favorable than that of the corresponding abasic product.[37] These results in conjunction with the data presented herein suggest that following initial Fpg recognition of a damaged base, glycosylase-mediated base excision creates a preferred Fpg ligand by producing an abasic site that permits greater surface area burial. Such an energetic landscape would ensure overall fidelity in the process of neutralizing potentially harmful and reactive nascent abasic sites.

Fpg binding to a gapped-duplex product mimic

In our effort to obtain a more complete picture of the energetic landscape of Fpg interactions with various intermediates in the catalytic process, we have assessed the interaction of Fpg with a 13-mer gapped duplex that serves as a product mimic. As one would normally expect, product release should be accompanied by a relative decrease in overall affinity for the product compared with the corresponding substrate. Our studies confirm that Fpg does not exhibit an appreciable affinity for the gapped DNA duplex, a finding that is entirely consistent with its catalytic function in terms of end product release. Although the product mimic lacks the 5′ phosphate group ordinarily present following δ-elimination (the latter identified in the complex as forming potential H-bonding interactions with Fpg amino acid side-chains),[9] the absence of interaction heats, Fpg protease protection, and band shift are consistent with kinetic studies,[34] whereby Fpg intrinsic fluorescence is restored upon product formation/release. It is noteworthy that the concerted action of several repair enzymes assumes an important role in preventing excessive exposure to intermediate abasic sites that are intrinsically toxic and among other consequences, may induce lethal strand breaks.[38,39] It is therefore not surprising that amongst the various transition state mimics studied for a number of repair systems, those resembling the abasic site transition state display the highest binding affinity, thereby enabling the repair enzymes to sequester these reactive species prior to removal/elimination.

Overall energetic-functional relationships

At present, there is no structural characterization of a catalytically functional glycosylase complexed with an 8-oxo-dG-containing DNA duplex, although Verdine and co-workers have reported a

structure for a complex containing a catalytically inactive mutant.[35] Co-crystallization of Fpg with lesion-containing duplexes has been performed either after adding reducing agent (i.e. $NaBH_4$) to entrap the Schiff base intermediate formed following base excision,[11] or complexed non-covalently with abasic site analogs such as the reduced abasic site,[10] or the opened ring 1,3 propanediol.[9] It is conceivable, however, that the structural features of the resultant complexes resemble the transition state comprising the abasic site-containing intermediate species, each structure representing a "snapshot" of an intermediate along this rather complex reaction coordinate. The heat capacity change measured for association of Fpg with the abasic site analogs suggests that the binding interaction is characterized by a large interface, since desolvation of hydrophobic surfaces is directly proportional to the magnitude of this thermodynamic parameter. The product of glycosylase activity (i.e. an abasic site) renders the lesion site a preferred ligand, presumably by increasing the potential for buried surface area, as well as reducing electrostatic repulsion and/or steric hindrance. As a result, glycosylase activity produces an enhanced substrate to ensure subsequent elimination reactions, thereby energetically favoring damaged base excision and formation of the gapped DNA product. For UDG·DNA interactions, mismatched base removal has been proposed to provide a more favorable geometry for subsequent interaction with the base-excision product,[37] a finding that is supported by our energetic data that demonstrate more favorable Fpg affinity for the abasic *versus* substrate analog.

We have designed future studies to evaluate the effects of ionic strength, buffer composition, and pH on the overall energetics of Fpg·DNA recognition. Systematic characterization of the energetics of Fpg·damaged DNA interactions as a function of ionic strength is of primary importance, particularly in view of the role that polyelectrolyte effects exert in terms of modulating protein–DNA interactions. In this regard, specific *versus* non-specific, hydrophobic *versus* electrostatic, and processive *versus* distributive mechanisms are variables that are affected by ionic strength, and therefore will be evaluated in the Fpg repair pathway. Nonetheless, our measurements have been performed under conditions that are comparable to those encountered *in vivo* amongst mesophilic organisms, particularly as far as ionic strength is concerned. It is important to note that even under these relatively sub-optimal ionic strength conditions that favor distributive *versus* processive mechanisms (J. Tchou & A.P.G., unpublished results on plasmid DNA), one still observes significant Fpg catalytic activity.

Concluding Remarks

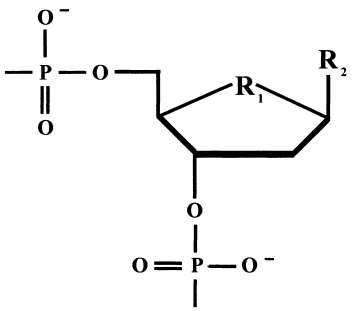
The present study furnishes a thermodynamic and extra-thermodynamic characterization of DNA recognition by Fpg, thereby providing the basis for an initial map of the energetic landscape of the recognition/repair "reaction coordinate". We have proposed interpretations of our macroscopic data in terms of microscopic contexts provided by recent crystal structures of complexes between Fpg and damaged DNA duplexes.[9–11] Parallel thermodynamic and crystallographic studies on such systems afford an opportunity to establish structure–function–energy correlations. Such correlations should lead to a better understanding of the molecular forces that modulate lesion recognition and repair. In support of this approach, it has been proposed that both structural and energetic consequences of damaged sites are exploited by repair enzymes to specifically target a particular lesion.[40–42] Studies have demonstrated that lesion-induced alterations of duplex energetics[40,41,43] may or may not be accompanied by significant structural variations from canonical duplex DNA.[32,40,41,44,45] Lesion-induced enthalpic destabilization[41,43] may contribute to reducing the energetic barrier associated with perturbation of DNA duplex structure in the resultant complex, consistent with an increased flexibility of most lesion-containing DNA duplexes.[46] The more subtle deviations of the 8-oxo-dG containing duplex from its corresponding counterpart, both in terms of energetic and structural aspects, suggests that Fpg specificity towards this lesion may reside largely in the catalytic term.

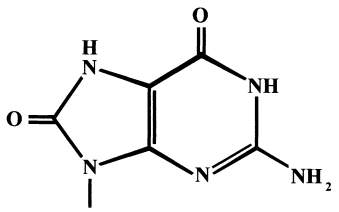
Our calorimetric results suggest that specific interactions of Fpg with a damaged base analog are minimal when compared with that of the abasic site, particularly under conditions of relatively high salt stringency. This observation, coupled with the absence of sequence specificity, might facilitate sliding of the protein along the substrate DNA when operating in the processive mode. Our energetic studies are consistent with this model, given that neither the canonical nor the carba-8-oxo-dG-containing DNA duplexes exhibit appreciable Fpg-binding affinity under the conditions employed herein. Studies currently in progress address the possible inequality of 8-oxo-dG and carba-8-oxo-dG with regards to Fpg-binding free energies and their underlying recognition mechanisms. It has been reported that the carba-8-oxo-dG derivative resists hydrolysis by several base excision repair enzymes[7] due to involvement of O4′ in the consecutive protonation–deprotonation events leading to formation of the aminal intermediate and resultant base excision. The implications of our results in conjunction with structural/biochemical evidence for mechanisms of damage recognition warrant further investigation.

Materials and Methods

Fpg expression and purification

Fpg was overexpressed and purified from B834 (DE3) *E. coli* harboring a PET13a-fpg plasmid, as described.[11]





8-oxo-2'-deoxyguanosine

Analog	R_1	R_2
8-oxo-dG	O	8-oxo-dG
carba-8-oxo-dG	CH_2	8-oxo-dG
tetrahydofuran (THF)	O	H
α-carba-abasic	CH_2	H

Scheme 2. Chemical structures of the analogs employed here.

Protein concentrations were determined by UV absorption spectroscopy using a calculated molar extinction coefficient of 39,410 M^{-1} cm^{-1} at 280 nm.

Oligonucleotide synthesis and purification

Synthesis of the undamaged deoxyoligonucleotide d(GCGATGGTTAGCG) and its complementary strand d(CGCTAACCATCGC) were performed on a model 8900 solid phase Expedite synthesizer (Applied Biosystems, Foster City, CA) using standard phosphoramidite chemistry. Specific lesions were introduced as the central base residue in the sequence d(GCGATGXTT AGCG), where X represents either THF, 8-oxo-dG, α-carba-AP, or carba-8-oxo-dG, with the latter two lesions involving an O4′ to CH_2 replacement in the deoxyribose ring. These modified deoxyoligonucleotides were synthesized and purified as described.[2,28,47] The identity and integrity of the damaged base-containing oligonucleotides (represented in Scheme 2) were assessed by mass spectrometry. Hexamers comprising the 5′ (i.e. d(GCGATG)) and 3′ (i.e. d(TTAGCG)) flanking sides of the lesion site were synthesized, purified, and annealed with the complementary 13-mer strand to mimic the end product of Fpg catalysis (except for the presence of a terminal phosphate group in the 5′ flanking sequence). Extinction coefficients for all of the deoxyoligonucleotides were determined by phosphate analysis.[48]

Isothermal titration microcalorimetry (ITC)

Calorimetric binding experiments were conducted on MicroCal model MSC-ITC and VP-ITC instruments (MicroCal LLC, Northampton, MA). The standard reaction buffer employed in the ITC experiments was comprised of 20 mM sodium phosphate, 200 mM sodium chloride, 1 mM dithiothreitol (DTT), adjusted to pH 7.0. Concentrated protein solutions were placed in an MWCO 10000 slide-a-lyzer membrane (Pierce Chemical Co., Rockford, IL) and dialyzed against four 1.0 l changes of buffer for 12 hours at 4 °C. To minimize oxidation of DTT throughout dialysis and during the course of the ITC experiments, the reaction buffer was prepared in the absence of DTT, cooled to 4 °C, and solid DTT added prior to each buffer exchange. The dialyzed protein and DNA stock solutions were diluted with an appropriate volume of dialyzate to yield final concentrations of 75.0 μM Fpg and 10.0 μM DNA in the titration syringe and ITC reaction cell, respectively. The deoxyoligonucleotide duplexes contained THF, carba-8-oxo-dG, or canonical guanosine as the central base in one strand forming a base-pair with the central cytosine base in the complementary strand. In a typical ITC experiment, the protein solution was titrated in 7.5 μl increments (total of 40 injections) at 300 second intervals into the DNA solution thermostated at the desired temperature. The quantity of heat absorbed upon Fpg binding to DNA was measured by integrating the area of each endotherm. Temperature-dependent changes in the binding isotherms were evaluated by conducting titration experiments at 5.0 °C, 10.0 °C, and 15.0 °C. A control experiment for dilution of protein into dialyzate in the absence of DNA was subtracted from each protein–DNA binding isotherm (data not shown). Dilution of dialyzate into DNA in the absence of protein produced negligible mixing artifacts. The resultant protein–DNA binding isotherms were analyzed using the Origin 5.0 software package (Origin, Northampton, MA) to extract the relevant thermodynamic parameters. The total heat (ΔH) and association constant (K_a) for the binding reaction were obtained by non-linear least-squares fitting of the data to a single-site binding model. Corrected binding enthalpies were plotted as a function of temperature, and the slope of the linear least-squares fit of the data yielded the heat capacity change (ΔC_p) of the protein–DNA interaction.

Circular dichroism

Circular dichroism spectropolarimetry (CD) was employed to assess Fpg conformation, and to evaluate the global structure of each of the oligonucleotide duplexes in the absence and presence of Fpg. Spectra were acquired on an AVIV model 62DS spectropolarimeter (AVIV Instruments, Lakewood, NJ) using a 1.0 mm quartz cuvette thermostated at 5.0 °C. Standard

solutions containing 10.0 μM Fpg, DNA or a 1:1 Fpg:DNA complex were scanned between 200 nm and 340 nm at 0.2 nm intervals employing a ten seconds averaging constant. The resultant CD spectra were normalized to units of mean residue molar ellipticity.

Protease protection assays

Protease digestions were employed to study the accessibility of Fpg within the complex compared to that of the free protein based on the assumption that the protein–DNA interface is protected from protease digestion. Fpg·DNA complexes were prepared at a ratio of 1:2 and incubated for 30 minutes at 20 °C prior to digestion. Endoproteinase Glu-C was added to the sample solutions at varying substrate:enzyme ratios (i.e. 5:1 to 50:1, w/w). Following incubation at 20 °C for 30 minutes, the reaction was terminated by addition of LDS sample buffer (NUPAGE, Invitrogen) and heated to 95 °C for three minutes. The digestion products were resolved in 10% Bis–Tris polyacrylamide gels under denaturing conditions. The protein bands were stained with Coomassie blue following gel electrophoresis, and the extent of digestion measured by densitometric analysis using an Alpha Innotech Imager.

Temperature-dependent UV spectroscopy

The DNA duplexes (1–150 μM) were prepared in the standard reaction buffer without DTT, placed in quartz cuvettes of different pathlengths (0.05–1.0 cm), and heated in the thermostated sample compartment of an AVIV model 14DS UV/Vis spectrophotometer (AVIV Instruments, Lakewood, NJ). The UV absorbance was monitored at 260 nm over the temperature range of 0–100 °C with measurements collected at 0.5 deg. C intervals employing a dwell time of 30 seconds and integration period of ten seconds. The resultant absorbance *versus* temperature profiles were analyzed to extract the duplex dissociation temperature (t_m).

Acknowledgements

We thank Professor Peter Privalov for useful discussions.

This work was supported by NIH grants GM23509, GM34469 and CA47995 (to K. J. B.). D. O. Z. acknowledges support from the Wellcome Trust (U.K.) and the Russian Foundation for Basic Research (02-04-49605).

References

1. David, S. S. & Williams, S. D. (1998). Chemistry of glycosylases and endonucleases involved in base-excision repair. *Chem. Rev.* **98**, 1221–1262.
2. Takeshita, M., Chang, C.-N., Johnson, F., Will, S. & Grollman, A. P. (1987). Oligodeoxynucleotides containing synthetic abasic sites. Model substrates for DNA polymerases and apurinic/apyrimidinic endonucleases. *J. Biol. Chem.* **262**, 10171–10179.
3. Tchou, J., Michaels, M. L., Miller, J. H. & Grollman, A. P. (1993). Function of the zinc finger in *Escherichia coli* Fpg protein. *J. Biol. Chem.* **268**, 26738–26744.
4. Castaing, B., Fourrey, J. L., Hervouet, N., Thomas, M., Boiteux, S. & Zelwer, C. (1999). AP site structural determinants for Fpg specific recognition. *Nucl. Acids Res.* **27**, 608–615.
5. Tchou, J. & Grollman, A. P. (1993). Repair of DNA containing the oxidatively-damaged base, 8-oxoguanine. *Mutat. Res.* **299**, 277–287.
6. O'Connor, T. R., Graves, R. J., de Murcia, G., Castaing, B. & Laval, J. (1993). Fpg protein of *Escherichia coli* is a zinc finger protein whose cysteine residues have a structural and/or functional role. *J. Biol. Chem.* **268**, 9063–9070.
7. Zharkov, D. O., Rieger, R. A., Iden, C. R. & Grollman, A. P. (1997). NH_2-terminal proline acts as a nucleophile in the glycosylase/AP-lyase reaction catalyzed by *Escherichia coli* formamidopyrimidine-DNA glycosylase (Fpg) protein. *J. Biol. Chem.* **272**, 5335–5341.
8. Sugahara, M., Mikawa, T., Kumasaka, T., Yamamoto, M., Kato, R., Fukuyama, K. *et al.* (2000). Crystal structure of a repair enzyme of oxidatively damaged DNA, MutM (Fpg), from an extreme thermophile, *Thermus thermophilus* HB8. *EMBO J.* **19**, 3857–3869.
9. Serre, L., Pereira De Jesus, K., Boiteux, S., Zelwer, C. & Castaing, B. (2002). Crystal structure of the *Lactococcus lactis* formamidopyrimidine-DNA glycosylase bound to an abasic site analogue-containing DNA. *EMBO J.* **21**, 2854–2865.
10. Fromme, J. C. & Verdine, G. L. (2002). Structural insights into lesion recognition and repair by the bacterial 8-oxoguanine DNA glycosylase MutM. *Nature Struct. Biol.* **9**, 544–552.
11. Gilboa, R., Zharkov, D. O., Golan, G., Fernandes, A. S., Gerchman, S. E., Matz, E. *et al.* (2002). Structure of formamidopyrimidine-DNA glycosylase covalently complexed to DNA. *J. Biol. Chem.* **277**, 19811–19816.
12. Privalov, P. L., Jelesarov, I., Read, C. M., Dragan, A. I. & Crane-Robinson, C. (1999). The energetics of HMG box interactions with DNA: thermodynamics of the DNA binding of the HMG box from mouse sox-5. *J. Mol. Biol.* **294**, 997–1013.
13. Vesnaver, G., Chang, C.-N., Eisenberg, M., Grollman, A. P. & Breslauer, K. J. (1989). The influence of abasic and anucleosidic sites on the stability, conformation, and melting behavior of a DNA duplex: correlations of thermodynamic and structural data. *Proc. Natl Acad. Sci. USA*, **86**, 3614–3618.
14. Sieber, M. & Allemann, R. K. (2000). Thermodynamics of DNA binding of MM17, a 'single chain dimer' of transcription factor MASH-1. *Nucl. Acids Res.* **28**, 2122–2127.
15. Spolar, R. S. & Record, M. T., Jr (1994). Coupling of local folding to site-specific binding of proteins to DNA. *Science*, **263**, 777–784.
16. Sturtevant, J. M. (1977). Heat capacity and entropy changes in processes involving proteins. *Proc. Natl Acad. Sci. USA*, **74**, 2236–2240.
17. Ha, J. H., Spolar, R. S. & Record, M. T., Jr (1989). Role of the hydrophobic effect in stability of site-specific protein–DNA complexes. *J. Mol. Biol.* **209**, 801–816.
18. Ladbury, J. E., Wright, J. G., Sturtevant, J. M. & Sigler, P. B. (1994). A thermodynamic study of the trp repressor–operator interaction. *J. Mol. Biol.* **238**, 669–681.
19. Carra, J. H. & Privalov, P. L. (1997). Energetics of folding and DNA binding of the MAT alpha 2 homeodomain. *Biochemistry*, **36**, 526–535.
20. Jen-Jacobson, L., Engler, L. E., Ames, J. T., Kurpiewski, M. R. & Grigorescu, A. (2000). Thermodynamic parameters of specific and nonspecific protein–DNA binding. *Supramol. Chem.* **12**, 143–160.

21. Takeda, Y., Ross, P. D. & Mudd, C. P. (1992). Thermodynamics of Cro protein–DNA interactions. *Proc. Natl Acad. Sci. USA*, **89**, 8180–8184.
22. Houbaviy, H. B. & Burley, S. K. (2001). Thermodynamic analysis of the interaction between YY1 and the AAV P5 promoter initiator element. *Chem. Biol.* **8**, 179–187.
23. Samland, A. K., Jelesarov, I., Kuhn, R., Amrhein, N. & Macheroux, P. (2001). Thermodynamic characterization of ligand-induced conformational changes in UDP-*N*-acetylglucosamine enolpyruvyl transferase. *Biochemistry*, **40**, 9950–9956.
24. Kozlov, A. G. & Lohman, T. M. (1999). Adenine base unstacking dominates the observed enthalpy and heat capacity changes for the *Escherichia coli* SSB tetramer binding to single-stranded oligoadenylates. *Biochemistry*, **38**, 7388–7397.
25. Jen-Jacobson, L., Engler, L. E. & Jacobson, L. A. (2000). Structural and thermodynamic strategies for site-specific DNA binding proteins. *Struct. Fold. Des.* **8**, 1015–1023.
26. Park, Y. W. & Breslauer, K. J. (1991). A spectroscopic and calorimetric study of the melting behaviors of a "bent" and a "normal" DNA duplex: $[d(GA_4T_4C)]_2$ *versus* $[d(GT_4A_4C)]_2$. *Proc. Natl Acad. Sci. USA*, **88**, 1551–1555.
27. Pilch, D. S., Xu, Z., Sun, Q., LaVoie, E. J., Liu, L. F. & Breslauer, K. J. (1997). A terbenzimidazole that preferentially binds and conformationally alters structurally distinct DNA duplex domains: a potential mechanism for topoisomerase I poisoning. *Proc. Natl Acad. Sci. USA*, **94**, 13565–13570.
28. Johnson, F., Dorman, G., Rieger, R. A., Marumoto, R., Iden, C. R. & Bonala, R. (1998). Synthesis of enzymatically noncleavable carbocyclic nucleosides for DNA-*N*-glycosylase studies. *Chem. Res. Toxicol.* **11**, 193–202.
29. Ishchenko, A. A., Bulychev, N. V., Maksakova, G. A., Johnson, F. & Nevinsky, G. A. (1999). Single-stranded oligodeoxyribonucleotides are substrates of Fpg protein from *Escherichia coli*. *IUBMB Life*, **48**, 613–618.
30. Gruskin, E. A. & Lloyd, R. S. (1986). The DNA scanning mechanism of T4 endonuclease V. Effect of NaCl concentration on processive nicking activity. *J. Biol. Chem.* **261**, 907–913.
31. Higley, M. & Lloyd, R. S. (1993). Processivity of uracil DNA glycosylase. *Mutat. Res.* **294**, 109–116.
32. Mol, C. D., Hosfield, D. J. & Tainer, J. A. (2000). Abasic site recognition by two apurinic/apyrimidinic endonuclease families in DNA base excision repair: the 3′ ends justify the means. *Mutat. Res.* **460**, 211–229.
33. Aida, M. & Nishimura, S. (1987). An *ab initio* molecular orbital study on the characteristics of 8-hydroxyguanine. *Mutat. Res.* **192**, 83–89.
34. Fedorova, O. S., Nevinsky, G. A., Koval, V. V., Ishchenko, A. A., Vasilenko, N. L. & Douglas, K. T. (2002). Stopped-flow kinetic studies of the interaction between *Escherichia coli* Fpg protein and DNA substrates. *Biochemistry*, **41**, 1520–1528.
35. Bruner, S. D., Norman, D. P. & Verdine, G. L. (2000). Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. *Nature*, **403**, 859–866.
36. Norman, D. P., Bruner, S. D. & Verdine, G. L. (2001). Coupling of substrate recognition and catalysis by a human base-excision DNA repair protein. *J. Am. Chem. Soc.* **123**, 359–360.
37. Parikh, S. S., Walcher, G., Jones, G. D., Slupphaug, G., Krokan, H. E., Blackburn, G. M. & Tainer, J. A. (2000). Uracil-DNA glycosylase-DNA substrate and product structures: conformational strain promotes catalytic efficiency by coupled stereoelectronic effects. *Proc. Natl Acad. Sci. USA*, **97**, 5083–5088.
38. Tchou, J., Bodepudi, V., Shibutani, S., Antoshechkin, I., Miller, J., Grollman, A. P. & Johnson, F. (1994). Substrate specificity of Fpg protein. Recognition and cleavage of oxidatively damaged DNA. *J. Biol. Chem.* **269**, 15318–15324.
39. Tchou, J., Kasai, H., Shibutani, S., Chung, M. H., Laval, J., Grollman, A. P. & Nishimura, S. (1991). 8-Oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. *Proc. Natl Acad. Sci. USA*, **88**, 4690–4694.
40. Plum, G. E. & Breslauer, K. J. (1994). DNA lesions: a thermodynamic perspective. *Ann. N.Y. Acad. Sci.* **726**, 45–56.
41. Plum, G. E., Grollman, A. P., Johnson, F. & Breslauer, K. J. (1995). Influence of the oxidatively damaged adduct 8-oxodeoxyguanosine on the conformation, energetics, and thermodynamic stability of a DNA duplex. *Biochemistry*, **34**, 16148–16160.
42. Sagi, J., Hang, B. & Singer, B. (1999). Sequence-dependent repair of synthetic AP sites in 15-mer and 35-mer oligonucleotides: role of thermodynamic stability imposed by neighbor bases. *Chem. Res. Toxicol.* **12**, 917–923.
43. Gelfand, C. A., Plum, G. E., Grollman, A. P., Johnson, F. & Breslauer, K. J. (1998). Thermodynamic consequences of an abasic lesion in duplex DNA are strongly dependent on base sequence. *Biochemistry*, **37**, 7321–7327.
44. Law, S. M., Erijita, R., Goodman, M. F. & Breslauer, K. J. (1996). Spectroscopic and calorimetric characterizations of DNA duplexes containing 2-aminopurine. *Biochemistry*, **35**, 12329–12337.
45. Rachofsky, E. L., Seibert, E., Stivers, J. T., Osman, R. & Ross, J. B. (2001). Conformation and dynamics of abasic sites in DNA investigated by time-resolved fluorescence of 2-aminopurine. *Biochemistry*, **40**, 957–967.
46. Lhomme, J., Constant, J. F. & Demeunynck, M. (1999). Abasic DNA structure, reactivity, and recognition. *Biopolymers*, **52**, 65–83.
47. Bodepudi, V., Shibutani, S. & Johnson, F. (1992). Synthesis of 2′-deoxy-7,8-dihydro-8-oxoguanosine and 2′-deoxy-7,8-dihydro-8-oxoadenosine and their incorporation into oligomeric DNA. *Chem. Res. Toxicol*, **5**, 608–617.
48. Snell, F. D. & Snell, C. T. (1972). *Colorimetric Methods of Analysis, Including Some Turbidimetric and Nephelometric Methods*, vol. 2, Van Nostrand, Huntington, New York.

Edited by K. Morikawa

(Received 16 January 2003; received in revised form 6 March 2003; accepted 7 March 2003)