Thermodynamics of the multi-stage DNA lesion recognition and repair by formamidopyrimidine-DNA glycosylase using pyrrolocytosine fluorescence—stopped-flow pre-steady-state kinetics

Nikita A. Kuznetsov¹, Yuri N. Vorobjev¹, Lev N. Krasnoperov² and Olga S. Fedorova¹,*

¹Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, 630090, Russia and ²New Jersey Institute of Technology, Newark NJ 07102, USA

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ABSTRACT

Formamidopyrimidine-DNA glycosylase, Fpg protein from Escherichia coli, initiates base excision repair in DNA by removing a wide variety of oxidized lesions. In this study, we perform thermodynamic analysis of the multi-stage interaction of Fpg with specific DNA-substrates containing 7,8-dihydro-8-oxoguanosine (oxoG), or tetrahydrofuran (THF, an uncleavable abasic site analog) and non-specific (G) DNA-ligand based on stopped-flow kinetic data. Pyrrolocytosine, highly fluorescent analog of the natural nucleobase cytosine, is used to record multi-stage DNA lesion recognition and repair kinetics over a temperature range (10–30°C). The kinetic data were used to obtain the standard Gibbs energy, enthalpy and entropy of the specific stages using van’t Hoff approach. The data suggest that not only enthalpy-driven exothermic oxoG recognition, but also the desolvation-accompanied entropy-driven enzyme-substrate complex adjustment into the catalytically active state play equally important roles in the overall process.

INTRODUCTION

Reactive oxygen species damage DNA to produce a variety of genotoxic lesions (1). In particular, 7,8-dihydro-8-oxoguanosine (oxoG) is one of the most common pre-mutagenic products of base oxidation in DNA (2). A variety of pathways for repair of damaged DNA exist (3). Among them, the predominant pathway is the base excision repair (BER) that processes small base lesions derived from the oxidation damage (4,5). DNA glycosylases play a key role in the BER pathway. They recognize a variety of modified or mismatched bases and release inappropriate bases from the deoxyribose phosphate chain (6,7).

OxoG is repaired through excision by formamidopyrimidine-DNA glycosylase (Fpg protein) in bacteria (8). Fpg protein possesses three types of activity: hydrolysis of the N-glycosidic bond with transient formation of an abasic (apurinic/apyrimidinic, AP) site (DNA glycosylase activity), elimination of the 3'-phosphate of the nascent AP-site (AP-lyase; β-elimination) and elimination of the 5'-phosphate of this modified AP-site (δ-elimination) (9). Consecutive execution of these three activities by Fpg removes the lesion from duplex DNA, where a single nucleotide gap in the damaged strand is left flanked by the phosphate residues. Catalysis by Fpg proceeds through the formation of a covalent imine (Schiff base) intermediate between Pro-1 residue of the enzyme and C-1' of the damaged nucleotide (10,11).

It is still unclear how DNA glycosylases efficiently select sparse lesions among the enormous excess of normal DNA (12–14). The three-dimensional structure of Escherichia coli Fpg shows that DNA binding is accompanied with drastic conformational changes, including DNA bending, eversion of oxoG from DNA and insertion of Met-73, Arg-108 and Phe-110 residues into DNA (15). Similar structural changes are observed in other DNA glycosylases (12).

One possibility is that the conformational transitions during the substrate binding occur sequentially, with at least some steps structurally optimized for the selection of correct substrates (14). This hypothesis is supported by the stopped-flow studies of E. coli Fpg protein that used tryptophan (Trp) and 2-aminopurine (2-aPu) as fluorescence reporters, which reveal several conformational changes preceding the irreversible steps in the reaction pathway (16–18).

The five-step binding kinetic scheme was revealed by the enzyme’s internal Trp fluorescence for oxoG-substrate recognition.
**Geobacillus stearothermophilus** 8-oxoguanine-DNA glycosylase, MutM enzyme from...ing rate constants of the forward and reverse reactions and...data. The multi-stage kinetic mechanism, the correspond-...action of Fpg with specific (oxoG, THF) and non-specific...bases, we perform thermodynamic analysis of the inter-...among an overwhelming number of undamaged DNA...the question of how glycosylases find single damages...checking for damages at random locations and to answer...[19] as the ‘phonograph needle’ mechanism[12] that permits...mechanism discussed above, where Phe-110 ‘wedge’ for-...taneously extruded lesion[14] in contrast to the ‘active’...glycosylase (UNG), in which the protein captures a spon-...from the ‘passive’ mechanism proposed for uracil DNA...Binding and cleavage of the oxoG/C-substrate by Fpg...Scheme 1. Binding and cleavage of the oxoG/C-substrate by Fpg...**

(E + S) \[ \overset{k_1}{\underset{k_i}{\rightarrow}} E \cdot S \overset{k_2}{\rightarrow} E \cdot S_2 \overset{k_3}{\rightarrow} E \cdot S_3 \overset{k_4}{\rightarrow} E \cdot S_4 \overset{k_5}{\rightarrow} E \cdot P \overset{k_s}{\rightarrow} E + P \]**

**Scheme 2. Binding and cleavage of the oxoG/C-substrate by Fpg** (2-αPu fluorescence detection).

(Scheme 1). The parallel analysis of 2-αPu fluorescence showed that the five reversible steps in Scheme 1 correspond to only two fluorescently discernible conformational changes in DNA (Scheme 2).

Additional information on the dynamics of Fpg protein was provided in the experiments with the F110W and F110A mutants[18] The substitution of Phe with a fluorescent Trp residue allowed identification of the reaction step(s) corresponding to the movement of Phe-110. It was shown that in the case of oxoG/C-substrate, the second step (τ1,2 of ca. 50 ms) and the third step (τ1,2 of ca. 500 ms) were specifically sensitive to this substitution which revealed the participation of Phe-110 in these recognition steps. These steps occur after the first step of non-specific binding but before the eversion of oxoG in the active site of the enzyme. On the fluorescence curves observed for the non-specific G/C-ligand, the second and the third steps are observed indicating participation of Phe-110 residue in the interrogation of lesions in DNA. Most likely, the second step represents insertion of Phe-110 in the DNA chain.[18] This interpretation has been corroborated recently in a single-molecule kinetics study.[19] It was suggested[18,20] that the third and fourth steps are eversion of oxoG in the Fpg active site, insertion of Met-73 and Arg-108 residues in the DNA void and DNA kink formation. The fifth step is the formation of a catalytically competent enzyme-substrate intermediate[18,20]. Previously, the active intrahelical interrogation and extrusion mechanism was proposed by Qi et al. [13] and Banerjee et al. [21] for a different bacterial 8-oxoguanine-DNA glycosylase, MutM enzyme from *Geobacillus stearothermophilus*. This mechanism differs from the ‘passive’ mechanism proposed for uracil DNA glycosylase (UNG), in which the protein captures a spontaneously extruded lesion[14] in contrast to the ‘active’ mechanism discussed above, where Phe-110 ‘ wedge’ formation is required in the lesion search.

To gain deeper insight in the mechanism [dubbed in[19] as the ‘phonograph needle’ mechanism] that permits checking for damages at random locations and to answer the question of how glycosylases find single damages among an overwhelming number of undamaged DNA bases, we perform thermodynamic analysis of the interaction of Fpg with specific (oxoG, THF) and non-specific (G) DNA-substrates based on the stopped-flow kinetic data. The multi-stage kinetic mechanism, the corresponding rate constants of the forward and reverse reactions and the resultant equilibrium constants are derived based on the temporal fluorescence traces of the fluorescent analog of a natural base, pyrrolocytosine, introduced in the DNA-substrates, over a very wide (six orders of magnitude, from milliseconds to thousand seconds) time and an extended temperature ranges. Pyrrolocytosine or 3-[b-D-2-ribofuranosyl]-6-methylpyrrolo[2,3-d]pyrimidin-2(3H)-one (Cpy), a fluorescent analog of the nucleoside cytidine retains its Watson–Crick base-pairing capacity with G and is only moderately affected by temperature, making it a versatile probe for fluorescent measurements of the kinetics at different temperatures[22].

The thermodynamic parameters of the specific stages obtained via the temperature-dependent study proved to be very helpful in the reliable identification of specific stages in the overall repair process based on the information that was not available earlier in the single temperature studies.

**MATERIALS AND METHODS**

**Oligodeoxynucleotides and enzymes**

Electrophoretically homogeneous *E. coli* Fpg protein was over-expressed, purified, quantified, assayed and stored as described previously.[15,17] The fraction of the active enzyme (~90%) was determined by borohydride trapping. The reaction mixture included 2 µM enzyme, 25 mM potassium phosphate (pH 6.8), 100 mM NaCl, 100 mM NaBH4 and varying amounts of oligonucleotide duplex containing an oxoG. The samples were incubated for 1 h at 25°C, mixed with the loading buffer [50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.1% bromophenol blue] and separated by 12% SDS-PAGE. The gel was stained with coomassie and quantified using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD, USA). Oligodeoxynucleotides d(TCTCTCTCXCCTTCCTT)/d(AAGGAAGGCGGCGAGAGAGA), where X is G, THF and oxoG, Cpy is fluorescent cytosine analogue pyrrolocytosine (Figure 1) were synthesized by established phosphoramidite methods on an ASM-700 synthesizer (Biosset Ltd., Novosibirsk, Russia) from phosphoramidites purchased from Glen Research (Sterling, VA, USA). The oligonucleotide containing oxoG was cleaved and deprotected with ammonium hydroxide containing 0.25 M 2-mercaptoethanol to avoid further oxidation of oxoG. Deprotected oligonucleotides were purified by ion-exchange HPLC on a Nucleosil 100-10 N(CH3)2 column followed by reverse-phase HPLC on a Nucleosil 100-7 C18 column (both 4.6 × 250 mm, purchased from Macherey-Nagel, Düren, Germany) to >98% homogeneity. The purity of oligonucleotides exceeded 98%, as estimated by electrophoresis in 20% denaturing...
polyacrylamide gel after staining with the Stains-All dye (Sigma-Aldrich, St. Louis, MO, USA). Concentrations of oligonucleotides were determined from their absorbance at 260 nm. Oligonucleotide duplexes were prepared by annealing modified and complementary strands at a 1:1 molar ratio in the reaction buffer [50 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 9% glycerol].

Stopped-flow measurements

Stopped-flow measurements with fluorescence detection were carried out using a model SX.18MV stopped-flow spectrometer (Applied Photophysics) as described in (16,17). All experiments were carried out in a buffer containing 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 9% glycerol.

Data processing

The approach is based on the fluorescence intensity variation in the course of the reaction due to the sequential formation and further transformation of DNA–enzyme complex and conformers. The kinetic parameters (both the rate constants and the response factors) were obtained by global non-linear fits using the DynaFit software (BioKin, Pullman, WA, USA) (23). The software performs numerical integration of a system of ODE with subsequent non-linear least-squares regression analysis. The response factors of the intermediates (that are essentially the products of the extinction coefficients and the fluorescence quantum yields) are treated as fitting parameters in the data processing.

In the evaluated mechanisms, except for the first bimolecular step, all other reactions are first order reactions. In the data processing, the kinetic information is obtained from the ‘temporal behavior’ of the fluorescence intensity, not from the ‘amplitudes’ of specific signal contributions. Only the rate constants obtained in non-linear fits were used to derive the thermochmical parameters. The ‘response factors’ for different conformers resulting from the fits were not used in the determination of the equilibrium constants, but rather provided additional information on the fluorescence intensity variations in different complexes and conformers.

Processing of individual kinetic curves does not unambiguously provide the kinetic parameters; therefore, global fits of sets of kinetic curves obtained at different concentrations of the reactants at each temperature was used. In the fits all relevant rate constants for the forward and reverse reactions, as well as the specific molar responses for all intermediate complexes were optimized.

Several mechanisms containing N binding reversible steps were evaluated. It was found that a binding mechanism with at minimum five reversible steps is required to provide fits with acceptable residuals comparable with the signal noise. The procedure of the mechanism determination was described in detail in previous publications (16,17).

Molecular modeling

The X-ray data obtained for the complexes of Fpg protein with identical 13-mer oxoG-DNA from Geobacillus stearothermophilus, pdb code 3GPP (13) and 3JR5 (24), respectively, are taken as models of atomic structures of protein–DNA complexes (E•OG), and (E•OG)5 in Scheme 1. Inspection of these structures revealed that the structure 3GPP represents an example of a non-specific protein–DNA complex (E•OG), with unbend DNA interacting with the protein over the minor groove. The 3JR5 represents specific protein–DNA complex (E•OG)5 with oxoG base everted from the double helix into the enzyme’s active site, with filled void in the double helix by Arg-112 and enzyme forming a tight complex with the DNA-substrate in the catalytically active conformation. The contact areas ΔMS (molecular surface) of the protein–DNA interface were calculated as the difference of the MS confining the solvent-excluded volume (25) of the isolated protein and DNA and protein–DNA complex, ΔMS = MS (protein–DNA complex) − MS (protein) − MS (DNA), where the buried MS has negative sign. The MS surface was calculated by the SIMS method (26). The free energy of the cavity formation in water solvent in the process of dissolution has entropic nature and is approximated by the linear equation ΔGcav = γΔMS, where the parameter γ is in the range of 70–117 cal/mol/Å2 (25). For estimation of the entropy loss we took the value of the parameter γ = 80 cal/mol/Å2 that approximates the entropy of dissolution of non-polar species in water (27).
observed at single-turnover conditions, indicating conformational transitions in the DNA molecule. In contrast to our previous studies (16–18), where 12-nt duplexes as DNA-substrates were studied, the longer DNA permitted investigation of the process over a wide temperature range (10–30°C).

The Cpy fluorescence traces were recorded for a set of Fpg concentrations at each temperature (10, 15, 20, 25, 30°C). The individual rate constants for all recognition steps under interaction with non-damaged DNA (G), with analog of AP-containing site (THF), and with oxoG-containing DNA (oxoG) were obtained from the fits.

The fluorescence of pyrrolocytosine is quenched in a double-stranded DNA compared with a single-stranded DNA (28,29). Figure 2A demonstrates, that the Cpy fluorescence intensity is larger for the enzyme–DNA complex (the middle part of the traces, 10–100 s) compared with the free double-stranded DNA (the initial parts of the traces, short times) and to the final non-specific DNA–product–enzyme complex formed after the removal of oxoG (the final part of the traces, long times). The increase of the plateau level with the increase of Fpg concentration (Figure 2A, long times) indicates that Cpy fluorescence intensity is higher when DNA is in the complex with the enzyme, compared with free dsDNA.

The measured fluorescence traces (Figure 2A) were fitted, as in the previous studies (16,17), by a kinetic mechanism (Scheme 1) containing five reversible steps, representing the sequential recognition of the damaged site and formation of the enzyme catalytically competent conformation. The sixth irreversible step corresponding to the catalytic reactions and the decomposition of the enzyme/product complex is clearly recognizable at high temperatures (Figure 3). The equilibrium constants were determined for each step in the reaction mechanism (Supplementary Table S1). The residuals of the fits by Scheme 1 are shown in Figure 2B. Figure 3 demonstrates the fluorescence traces obtained for 1 μM Fpg and 1 μM oxoG/Cpy-containing DNA-substrate at different temperatures.

To detect the fluorescence changes Cpy responsible to non-specific dsDNA binding, we used an undamaged duplex (G-ligand) (Figure 4A). The process of binding the non-specific G-ligand was completed by 0.1 s. An increase in the fluorescence intensity of Cpy was observed indicating destabilization of Watson–Crick or stacking interactions in the primary non-specific enzyme–DNA complex. The ‘melting’ of DNA structure was shown in our previous work using undamaged duplex with 2-aPu residue (17). According to Qi et al., studies (24) three amino acid residues [Arg-77, Met-112 and Phe-114 for Fpg from G. stearothermophilus, which are analogs of Met-73, Arg-108 and Phe-110 in Fpg from E. coli] penetrate DNA duplex and stabilize the bended DNA in non-specific complex. In this complex normal G base is partially extruded from the DNA, but still does not attain the catalytic site of the enzyme. In the recent work of Dunn et al. (19) it was shown that Phe-111 residue of E. coli Fpg (corresponds to Phe-110 in our work) might play a crucial role in the base interrogation. Measuring the rate of the enzyme scanning along DNA, it was found that when this residue was mutated in the E. coli Fpg gene to an alanine, there was a dramatic increase in the overall diffusion rate of the variant compared with the wild-type protein.

Fitting the experimental data to the one-step binding model (Scheme 3) gave the values for the forward and reverse rate constants and dissociation constant at different temperatures (Supplementary Table S2).

To gain a better understanding of the recognition of specific substrate by E. coli Fpg protein, we used the
model duplex containing the non-cleavable abasic site analog (THF-ligand). THF moiety (Figure 1) lacks the C-1' hydroxyl group of the 2'-deoxyribose. This feature allows elimination of the catalytic event that occurs subsequent to abasic site recognition and to use THF-ligand for specific recognition studies. According to the X-ray data, the DNA in this complex is kinked at the point of the lesion, and Met-73, Arg-108 and Phe-110 are inserted in the DNA to fill the abasic void (30).

The changes in the CPy fluorescence during the interaction of Fpg with (A) G- and (B) THF-ligand at different temperatures. The concentrations of the enzyme and the DNA were 1 μM.

![Figure 4. Changes in the CPy fluorescence intensity during the interaction of Fpg with (A) G- and (B) THF-ligand at different temperatures.](image)

Scheme 3. Binding of the G-ligand by Fpg (CPy fluorescence detection).

Using measured rate constants the equilibrium constants $K_i$ ($k_i/k_{-i}$, $i$ is the step number) were determined for G- and THF-ligands and oxoG-substrate. The $\Delta H^o_i$ and $\Delta S^o_i$ were calculated using the relationship: $ln(K_i) = \Delta S^o_i/R - \Delta H^o_i/RT$. As shown in Figure 5A, the dependence $ln(K_i)$ versus 1/T is linear, as expected for the relatively narrow temperature range of the study. The Gibbs free energies $\Delta G^o_i$ at 25°C were calculated from $ln(K_i) = -\Delta G^o_i/RT$ (Table 1). Visualization of the inter-relationship of the thermodynamic parameters of the binding steps of oxoG-substrate is presented in Figure 5B.

Inspection of the thermodynamic data summarized in Table 1 reveals a clear qualitative difference in the

| Binding steps | \( \Delta G^o_1 \) | \( \Delta G^o_2 \) | \( \Delta G^o_3 \) | \( \Delta G^o_4 \) | \( \Delta G^o_5 \) |
|--------------|----------------|----------------|----------------|----------------|----------------|
| 1            | -2.0 | -1.5 | -1.0 | -0.5 | 0.0 |
| 2            | -1.5 | -1.0 | -0.5 | 0.0 | 0.5 |
| 3            | -1.0 | -0.5 | 0.0 | 0.5 | 1.0 |
| 4            | -0.5 | 0.0 | 0.5 | 1.0 | 1.5 |
| 5            | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 |

Table 1. Thermodynamic parameters of the binding steps of oxoG-substrate.
thermodynamics of Fpg binding to non-specific and specific DNA substrates that is important for the understanding of the mechanisms of specific sites recognition by the DNA glycosylases.

The first step of oxoG-recognition has similar thermodynamic parameters as the binding of non-specific DNA and the first step of THF-ligand. The Gibbs free energy of binding (ca. 8 kcal/mol) is typical for protein–DNA complexes (31). Formation of the protein–DNA complex restraints translational, rotational and conformational motions of the protein and DNA leading to the entropy loss. The entropy gain is provided by water molecules release from the protein–DNA interface (31), which has been shown to accompany molecular complexation in many systems, including protein–DNA complexes.

To analyze the relationship between species in Schemes 1 and 2 registered by the tryptophan (16), 2-aminopurine (17) and pyrrolocytosine fluorescence, we performed a kinetic simulation of the reaction pathway using the rate constants determined by detection of all fluorescence types for the oxoG-substrate, and compared the time courses of the formation and disappearance of the intermediate species (Figure 6).

The comparison of the characteristic times of the appearance and disappearance of various enzyme–DNA complexes (Figure 6) shows that the first step of binding leads to simultaneous formation of ES₁ and ES₂. However, conversion of ES₁ to ES₂ occurs faster than the conversion of ES₁ to ES₂ indicating that the conformational changes of the enzyme occur before the binding with the DNA-specific site. This observation is in good agreement with the data (18,19) suggesting an

### Table 1. Thermodynamics parameters of Fpg interactions with DNA

| DNA Step number | ΔG<sub>i298</sub>, kcal/mol | ΔH<sub>i</sub>, kcal/mol | ΔS<sub>i</sub>, cal/K*mol | Equilibrium constants Kᵢ (298K) | Process |
|-----------------|-----------------------------|--------------------------|--------------------------|---------------------------------|---------|
| G               | −7.0                        | −3.8 ± 0.9               | 10.9 ± 3.2               | 1.2 × 10⁵ M⁻¹                  | Non-specific binding, DNA distortion |
| THF             | −7.2                        | −4.0 ± 0.3               | 10.8 ± 1.0               | 1.9 × 10⁵ M⁻¹                  | Non-specific binding, DNA distortion |
| THF             | 0.7                         | 6.7 ± 0.3                | 20.3 ± 0.9               | 0.5                             | DNA kinking together with amino acids insertion |
| oxoG            | −7.0                        | −3.2 ± 0.4               | 12.7 ± 1.5               | 1.4 × 10⁵ M⁻¹                  | Non-specific binding, DNA distortion |
| oxoG            | 0.4                         | 0.3 ± 0.8                | −0.3 ± 2.7               | 0.5                             | Enzyme conformational change into interrogation capable Phe-110 ‘wedge’ state |
| oxoG            | 0.8                         | 6.3 ± 1.7                | 18.4 ± 5.8               | 0.25                            | DNA kinking |
| oxoG            | −1.5                        | −15.5 ± 3.9              | −46.9 ± 13.5             | 12.7                            | Eversion of oxoG into enzyme active center together with Arg-108 and Met-73 insertion |
| oxoG            | 0.8                         | 31.2 ± 5.5               | 111.1 ± 18.6             | 25.0                            | Final adjustment of enzyme active center, DNA desolvation |
| oxoG            | −1.9                        | 31.2 ± 5.5               | 111.1 ± 18.6             | 25.0                            | Irreversible catalytic step |

The errors indicated are ±1 SD ΔΔG<sub>i298</sub> = RT(ΔKᵢ/Kᵢ) ≤ 0.1 kcal/mol.

Apparent thermodynamic parameters of the ‘transition state’ calculated as ΔG<sub>st</sub> = RT ln(kᵦT/hkᵦ), where kᵦ and h are Boltzmann and Planck’s constants, respectively, R is the gas constant, T is absolute temperature in Kelvins.

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![Figure 6](image-url)
active mechanism of the lesion search by Phe-110 at the first step of recognition. It should be noted that the formation of the ESi complexes detected by CPy fluorescence occurs after the formation of corresponding ESi complexes detected by Trp fluorescence, whereas only two complexes were detected by measurements of 2-aPu fluorescence. The ES1 2-aPu likely corresponds to ES2 and ES3, whereas ES2 2-aPu corresponds to ES4 and ES5, both detected by the Trp and CPy fluorescence.

We suggested that formation of ES2 2-aPu complex leads to destabilization of the local DNA structure, this process also detected by CPy fluorescence with a delay of a few millisecond (formation of ESi CPy). At this stage, 2-aPu fluorescence indicates a decrease in the level of stacking of the 2-aPu reporter base adjacent to the damaged base. The next step of the recognition accompanied with the formation of ES1 complex, was attributed to the bending of the DNA chain. The ES1 complex was ascribed to the oxoG eversion and the amino acids plugging into the DNA chain because of the decrease in the 2-aPu and CPy fluorescence intensity in this step. In the last recognition step detected by Trp and CPy fluorescence all specific contacts are formed in the complex ES3 and the next catalytic reaction step can proceed.

The thermodynamic parameters of the steps of oxoG recognition, together with our previous data (16–18) on the Trp and 2-aminopurine fluorescence changes upon Fpg complexation with DNA, suggest that the sequential reaction steps in Scheme 1 correspond to: (i) non-specific primary encounter; (ii) initial recognition with the destabilization of the DNA around the lesion with the insertion of Phe-110; (iii) formation of a kink in the DNA chain; (iv) eversion of oxoG base from the double helix into the enzyme’s active site; filling the resulting void in the double helix by Arg-108 and Met-73 and (v) isomerization of the enzyme to form a tight complex with the DNA-substrate and to produce catalytically active conformation.

The first step has similar thermodynamic parameters for all DNA ligands containing G, THF and oxoG. In this step the moderate enthalpy gain is accompanied by an increase in entropy most probably due to the DNA ‘melting’ in the point of contact and the conformational changes of the DNA binding site in the Fpg molecule with the emphasis on the contribution of the Phe-110 wedge movement (13,16–19).

The second step is energetically neutral that is very important for the ‘active’ mechanism of the lesion chasing using the Phe-110 ‘wedge’.

The third step is endothermic but is accompanied by the increase in entropy. This is consistent with the energy required to kink the DNA and the water molecules release in the kink position.

The fourth step has favorable enthalpy but unfavorable entropy, which makes us to modify our previous interpretation (18,20) and to assign this step to extrusion of oxoG base from the DNA chain and the insertion in the enzyme active site, as well as the insertion of Arg-108 and Met-73 residue in the DNA chain (Figure 7). In this step, new contacts between aminoacids of the active center and oxoG residue, as well as between the aminoacids of Fpg with the DNA in the void are formed that leads to the energy gain. However, the resulting structure is more rigid, which explains the entropy loss.

The fifth reversible recognition step is characterized by an unfavorable enthalpy that is compensated by favorable entropic contribution. The large positive entropy of protein binding originates from the dehydration of the DNA grooves. Changes in the solvation are crucial in assisting the site discrimination during the catalysis, as well as in the directing sequence-specific DNA binding by numerous enzymes [see, e.g. (31)].

Using X-ray data obtained for the complexes of Fpg protein from G. stearothermophilus with oxoG-DNA, [pdb code 3GPP (13) and 3JR5 (24)] as atomic models for the Fpg–DNA complexes of the Stage 1 and Stage 5, respectively, change of the area of the protein–DNA interface during the formation of the catalytically competent complex ES5 from the initial non-specific complex ES1 was estimated. The DNA bending and the insertion of the oxoG into protein cavity in the complex ES5 are accompanied by the increase of the contact surface interface by 680 Å² (from 894 to 1583 Å² in complexes ES1 and ES5, respectively). Up to five additional base pairs of DNA are involved in the contact with the protein over the major groove of DNA. Burring of 680 Å² of the molecular surface of the tight protein–DNA complex gives entropy gain of about 180 cal/K*mol. This number is in a qualitative agreement with the data in Table 1, taking into account that formation of the tight specific protein–DNA complex leads to a decrease of the conformational entropy of the complex.

Total change of Gibbs energy, \( \Delta G_{298} = \Sigma \Delta G_i = -9.2 \text{ kcal/mol} \) within the experimental error agrees with our previous value of \(-10.9 \text{ kcal/mol} \) obtained using the ‘stepwise increased ligand complexity’ (SILC) method (32). The energies of the first steps of oxoG-substrate
and G- and THF-ligands binding, as well as the product dissociation step are in good agreement. It means that this stage of binding is characterized by the same processes that are independent of the different structure of DNA duplexes. Our data obtained for the binding of the THF-ligand are in agreement with duplexes. Our data obtained for the binding of the Fpg protein with oxoG-containing DNA substrate differs from the data obtained for the interaction of Fpg protein with the THF-ligand, which model the apurinic/apyrimidinic site, the intermediate of oxoG conversion. Comparison of these values leads to the conclusion that recognition of oxoG residue versus AP-site has unfavorable enthalpy (\(\Delta H = 16.4 \text{ kcal/mol}\) but favorable entropy (\(\Delta S = 63.9 \text{ cal/K*mol}\)). This difference can be explained by the very unfavorable enthalpy of adjustment of enzyme–DNA complex to catalytically competent state. On the other hand, the binding of oxoG-containing duplex leads to the formation of additional contacts in the protein–DNA complex. This induces additional desolvation of the protein and DNA surfaces that increases entropy. Therefore, essentially the desolvation-related entropic forces drive protein into the specific binding with DNA and to adjustment to the catalytically competent conformation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Experimental Data.

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REFERENCES

1. von Sonntag,C. (2006) Free-Radical-Induced DNA Damage and Its Repair: A Chemical Perspective. Springer, Berlin - Heidelberg.
2. Wallace,S.S. (2002) Biological consequences of free radical-damaged DNA bases. Free Radic. Biol. Med., 33, 1–14.
3. Friedberg,E.C., Walker,G.C. and Siede,W. (1995) DNA Repair and Mutagenesis. ASM Press, Washington.
4. Gros,L., Sarapbaev,M.K. and Laval,J. (2002) Enzymology of the repair of free radicals-induced DNA damage. Oncogene, 21, 8905–8925.
5. Memisoglu,A. and Samson,L. (2000) Base excision repair in yeast and mammals. Mutat. Res., 451, 39–51.
6. David,S.S. and Williams,S.D. (1998) Chemistry of glycosylases and endonucleases involved in base-excision repair. Chem. Rev., 98, 1221–1261.
7. Krokan,H.E., Standal,R. and Slupphaug,G. (1997) DNA glycosylases in the base excision repair of DNA. Biochem. J., 325, 1–16.
8. Zharkov,D.O., Shoham,G. and Grollman,A.P. (2003) Structural characterization of the Fpg family of DNA glycosylases. DNA Repair, 2, 839–862.
9. Bhagwat,M. and Gerlt,J.A. (1996) 3'- and 5'-strand cleavage reactions catalyzed by the Fpg protein from Escherichia coli occur via successive \(\beta-\) and \(\delta-\)elimination mechanisms, respectively. Biochemistry, 35, 659–665.
10. Tchou,J. and Grollman,A.P. (1995) The catalytic mechanism of Fpg protein. Evidence for a Schiff base intermediate and amino terminus localization of the catalytic site. J. Biol. Chem., 270, 11671–11677.
11. Zharkov,D.O., Rieger,R.A., Iden,C.R. and Grollman,A.P. (1997) NH2-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.
12. Hitomi,K., Iwai,S. and Tainer,J.A. (2007) The intricate structural chemistry of base excision repair machinery: implications for DNA damage recognition, removal, and repair. DNA Repair, 6, 410–428.
13. Qi,Y., Spong,M.C., Nam,K., Banerjee,A., Jiralerspong,S., Karplus,M. and Verdine,G.L. (2009) Encounter and extrusion of an intrahelical lesion by a DNA repair enzyme. Nature, 462, 762–766.
14. Friedman,J.I. and Stivers,J.T. (2010) Detection of damaged DNA bases by DNA glycosylase enzymes. Biochemistry, 49, 4957–4967.
15. Gilboa,R., Zharkov,D.O., Golan,G., Fernandes,A.S., Gerchman,S.E., Matz,E., Kycia,J.H., Grollman,A.P. and Shoham,G. (2002) Structure of formamidopyrimidine-DNA glycosylase covalently complexed to DNA. J. Biol. Chem., 277, 19811–19816.
16. Koval,V.V., Kuznetsova,N.A., Zharkov,D.O., Ishchenko,A.A., Douglas,K.T., Nevisky,G.A. and Fedorova,O.S. (2004) Pre-steady-state kinetics shows differences in processing of various DNA lesions by Escherichia coli formamidopyrimidine-DNA glycosylase. Nucleic Acids Res., 32, 926–935.
17. Kuznetsova,N.A., Koval,V.V., Zharkov,D.O., Vorobiev,Y.N., Nevisky,G.A., Douglas,K.T. and Fedorova,O.S. (2007) Kinetic basis of lesion specificity and opposite-base specificity of Escherichia coli formamidopyrimidine-DNA glycosylase. Biochemistry, 46, 424–435.
18. Koval,V.V., Kuznetsova,N.A., Ishchenko,A.A., Sarapbaev,M.K. and Fedorova,O.S. (2010) Real-time studies of conformational dynamics of the repair enzyme E. coli formamidopyrimidine-DNA glycosylase and its DNA complexes during catalytic cycle. Mutat. Res., 685, 3–10.
19. Dunn,A.R., Kad,N.M., Nelson,S.R., Warshaw,D.M. and Wallace,S.S. (2011) Single Qdot-labeled glycosylase molecules use a wedge amino acid to probe for lesions while scanning along DNA. Nucleic Acids Res., 39, 7487–7498.
20. Fedorova,O.S., Kuznetsova,N.A., Koval,V.V. and Knoorre,D.G. (2010) Conformational dynamics and pre-steady-state kinetics of DNA glycosylases. Biochemistry (Moscow), 75, 1225–1239.
21. Banerjee,A., Santos,W.L. and Verdine,G.L. (2006) Structure of a DNA glycosylase searching for lesions. Science, 311, 1153–1157.
22. Tinsley,R.A. and Walter,N.G. (2006) Pyrrolo-C as a fluorescent probe for monitoring RNA secondary structure formation. RNA, 12, 522–529.
23. Kuzmic,P. (1996) Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV protease. Anal. Biochem., 237, 260–273.
24. Qi,Y., Spong,M.C., Nam,K., Karplus,M. and Verdine,G.L. (2010) Entrapment and structure of an extrahelical guanine attempting to enter the active site of a bacterial DNA glycosylase, MutM. J. Biol. Chem., 285, 1468–1478.
25. Vorobjev,Y.N. (2011) Advances in implicit models of water solvent to compute conformational free energy and molecular dynamics of proteins at constant pH. Adv. Protein Chem. Struct. Biol., 85, 281–322.
26. Vorobjev, Y.N. and Hermans, J. (1997) SIMS: computation of a smooth invariant molecular surface. *Biophys. J.*, **73**, 722–732.
27. Rose, G.D. and Wolfenden, R. (1993) Hydrogen bonding, hydrophobicity, packing, and protein folding. *Annu. Rev. Biophys. Biomol. Struct.*, **22**, 381–415.
28. Rist, M.J. and Marino, J.P. (2002) Fluorescent nucleotide base analogs as probes of nucleic acid structure, dynamics and interactions. *Curr. Org. Chem.*, **6**, 775–793.
29. Berry, D.A., Jung, K.Y., Wise, D.S., Sercel, A.D., Pearson, W.H., Mackie, H., Randolph, J.B. and Somers, R.L. (2004) Pyrrolo-dC and pyrrolo-C: fluorescent analogs of cytidine and 2′-deoxycytidine for the study of oligonucleotides. *Tetrahedron Lett.*, **45**, 2457–2461.
30. Pereira de Jesus, K., Serre, L., Zelwer, C. and Castaing, B. (2005) Structural insights into abasic site for Fpg specific binding and catalysis: comparative high-resolution crystallographic studies of Fpg bound to various models of abasic site analogues-containing DNA. *Nucleic Acids Res.*, **33**, 5936–5944.
31. Privalov, P.L., Dragan, A.I., Crane-Robinson, C., Breslauer, K.J., Remeta, D.P. and Minetti, C.A. (2007) What drives proteins into the major or minor grooves of DNA? *J. Mol. Biol.*, **365**, 1–9.
32. Ishchenko, A.A., Vasilenko, N.L., Sinitsina, O.I., Yamkovoy, V.I., Fedorova, O.S., Douglas, K.T. and Nevinsky, G.A. (2002) Thermodynamic, kinetic, and structural basis for recognition and repair of 8-oxoguanine in DNA by Fpg protein from *Escherichia coli*. *Biochemistry*, **41**, 7540–7548.
33. Minetti, C.A., Remeta, D.P., Zharkov, D.O., Plum, G.E., Johnson, F., Grossman, A.P. and Breslauer, K.J. (2003) Energetics of lesion recognition by a DNA repair protein: thermodynamic characterization of formamidopyrimidine-glycosylase (Fpg) interactions with damaged DNA duplexes. *J. Mol. Biol.*, **328**, 1047–1060.