Pre-steady-state kinetics shows differences in processing of various DNA lesions by *Escherichia coli* formamidopyrimidine-DNA glycosylase

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ABSTRACT

Formamidopyrimidine-DNA-glycosylase (Fpg protein, MutM) catalyses excision of 8-oxoguanine (8-oxoG) and other oxidatively damaged purines from DNA in a glycosylase/apurinic/apyrimidinic-lyase reaction. We report pre-steady-state kinetic analysis of Fpg action on oligonucleotide duplexes containing 8-oxo2'-deoxyguanosine, natural abasic site or tetrahydrofuran (an uncleavable abasic site analogue). Monitoring Fpg intrinsic tryptophan fluorescence in stopped-flow experiments reveals multiple conformational transitions in the protein molecule during the catalytic cycle. At least four and five conformational transitions occur in Fpg during the interaction with abasic and 8-oxoG-containing substrates, respectively, within 2 ms to 10 s time range. These transitions reflect the stages of enzyme binding to DNA and lesion recognition with the mutual adjustment of DNA and enzyme structures to achieve catalytically competent conformation. Unlike these well-defined binding steps, catalytic stages are not associated with discernible fluorescence events. Only a single conformational change is detected for the cleavable substrates at times exceeding 10 s. The data obtained provide evidence that several fast sequential conformational changes occur in Fpg after binding to its substrate, converting the protein into a catalytically active conformation.

INTRODUCTION

Reactive oxygen species (ROS), including hydrogen peroxide, superoxide anion radical, hydroxyl radical and singlet oxygen, are formed under conditions of oxidative stress, UV or ionizing irradiation, and during normal cell metabolism (1,2). ROS have been implicated in mutagenesis, carcinogenesis and ageing (2) and can induce formation of DNA base lesions, such as 8-oxoguanine (8-oxoG), thymine glycols, formamidopyrimidine derivatives of guanine (2,6-diamino-4-hydroxy-5-formamidopyrimidine, Fapy-G) and adenine (4,6-diamino-5-formamidopyrimidine, Fapy-A), and many others (1). 8-OxoG may ultimately result in a G:C→T:A transversion (3–5). In vitro, both Fapy-G and Fapy-A also promote misincorporation of adenine opposite the lesion by Klenow fragment of *Escherichia coli* DNA polymerase I (6,7). To deal with such problems, a special system for repair of 8-oxoG and Fapy has evolved. In *E.coli*, damaged purines, including Fapy lesions and 8-oxoG (8–11), are removed from DNA by formamidopyrimidine-DNA glycosylase (Fpg, also known as MutM). 8-OxoG is believed to be the most physiologically relevant substrate for this enzyme (8), although recent data suggest that the removal of Fapy residues by Fpg may be of equal importance (12,13). Fpg 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; this reaction is usually referred to as β-elimination) and elimination of the 5'-phosphate of thus modified AP site (a reaction often termed δ-elimination) (14,15). 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 phosphate residues. Catalysis by Fpg proceeds through formation of a covalent imine (Schiff base) intermediate between Pro-1 residue of the enzyme and C1' of the damaged nucleotide (16,17).

Many structural features of the interactions of Fpg with damaged DNA are known from X-ray crystallographic studies (18–21). Fpg consists of two domains connected by a hinge polypeptide. The N-terminal domain contains a β-sandwich core and a long α-helix with an N-terminal catalytic dyad proline-glutamate. The C-terminal domain is mostly α-helical, containing two motifs almost universally conserved in Fpg proteins: a helix–two turn–helix (H2TH) motif and a β-hairpin Cys4 zinc finger. The protein molecule possesses a positively charged cleft where damaged DNA is bound. For catalysis to occur the damaged base must be extruded from the DNA helix and placed in the active site of enzyme; this is achieved in Fpg...
by kinking DNA at the lesion point. This ‘flip-out’ step is a common feature for the majority of DNA glycosylases (22).

Despite the fact that the structure of the Fpg–DNA complex is established, little is known about the dynamics of substrate recognition by this enzyme. This paucity of data on recognition is further exacerbated by the fact that all Fpg–DNA structures obtained so far lack the damaged base, reflecting steps after base excision. Using steady-state kinetic methods to assess the contribution of different parts of the substrate molecule to binding, we have shown that Fpg forms weak non-specific bonds with almost all nucleotide pair units accommodated within its DNA-binding cleft (23–25). These interactions provide about seven orders of magnitude of the enzyme’s affinity for DNA in total, with the relative contribution of the specific interactions (i.e. with 8-oxoG) being less than two orders of magnitude. Formation of the Michaelis complex between Fpg and 8-oxoG-containing DNA cannot by itself account for the major part of the enzyme specificity, which lies rather in the $k_{\text{cat}}$ term: the rate increases by 6–8 orders of magnitude on transition from non-specific to specific oligodeoxyribonucleotides. Therefore, conformational adjustment of the enzyme and the substrate to provide proper orbital alignment for catalysis is of the utmost importance for Fpg specificity. This finding is not unique for Fpg but holds for other DNA repair enzymes, such as uracil-DNA glycosylase or AP endonuclease (26).

Recently, we applied a stopped-flow approach to address the kinetic mechanism of 8-oxoG excision from DNA by Fpg (27). Using a duplex 8-oxoG-containing dodecamer as a substrate and following the enzyme’s internal tryptophan fluorescence, we detected and kinetically resolved multiple conformational changes in Fpg protein during the catalytic process. Towards better understanding of the nature of each phase we have now examined the effects of structural variations at the lesion site on the kinetics of Fpg action. We used specific substrates and non-cleavable ligands containing either 8-oxoG, a natural AP site, or tetrahydrofuran residues (F, a non-cleavable AP site analogue; Fig. 1). A careful examination of fluorescence traces in several critical time-course segments allowed us to identify and assign several additional conformational changes in Fpg during the process of catalysis. We now provide evidence that the rate-determining step corresponds to catalytic rate constant $k_{\text{cat}}$ in Michaelis–Menten equation under steady-state conditions.

**MATERIALS AND METHODS**

**Oligodeoxyribonucleotides and enzymes**

Electrophoretically homogeneous Fpg protein was over-expressed, purified, quantified, assayed and stored as described previously (23). Uracil-DNA glycosylase was purchased from New England Biolabs (Beverly, MA). Oligodeoxyribonucleotides d(GGAAGGCGAGAG), d(CTCTCCp), d(pCCTTCC) and d(CTCTCXCCTTCC), where X is 8-oxoG, deoxyuridine (U) or F, were synthesized by established phosphoramidite methods on an ASM-700 synthesizer (BIOSSET Ltd, Novosibirsk, Russia) from synthesizer (BIOSSET Ltd, Novosibirsk, Russia) from phosphoramidites purchased from Glen Research (Sterling, VA). The oligonucleotide containing 8-oxoG was cleaved and deprotected with ammonium hydroxide containing 0.25 M 2-mercaptoethanol to avoid further oxidation of 8-oxoG.

Deprotected oligonucleotides were purified by ion-exchange HPLC on a Nucleosil 100-10 N(CH$_3$)$_2$ column followed by reverse-phase HPLC on a Nucleosil 100-7 C$_{18}$ column (both 4.6 × 250 mm, purchased from Macherey-Nagel, Düren, Germany). 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). Concentrations of oligonucleotides were determined from their absorbance at 260 nm. The AP-containing oligonucleotide was prepared as described (28) by incubating the deoxyuridine-containing oligonucleotide (0.1 µmol) for 14 h at 37°C with 15 U of uracil-DNA glycosylase in 150 µl of 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin. Reverse phase HPLC on the Nucleosil 100-7 C$_{18}$ column was used to purify the reaction product, which had shorter retention time than the starting oligonucleotide, using a linear gradient of 0–20% acetonitrile in 0.1 M triethylammonium acetate (pH 7.0). The pooled fractions were concentrated and then converted to the lithium salt form using a Sep-Pak Plus C$_{18}$ cartridge (Waters, Milford, MA). The integrity of AP-containing oligonucleotides was assessed by electrophoresis followed by staining, as described above. To confirm the presence of the AP site in the oligonucleotide after treatment with uracil-DNA glycosylase, the samples of the oligonucleotide were treated with 10% aqueous piperidine at 95°C, or annealed to the complementary oligonucleotide and treated with Fpg under conditions described below. Polyacrylamide gel electrophoresis indicated that in both cases the material was cleaved to two shorter oligonucleotides of different lengths. Oligonucleotides containing no AP site or 8-oxoG remained intact after the same treatment.

**Stopped-flow studies**

Stopped-flow fluorescence rate measurements were carried out essentially as described (27) using a model SX.18MV stopped-flow spectrometer (Applied Photophysics, UK) fitted with a 150 W Xe arc lamp with excitation at 293 nm. All experiments were carried out at 25°C in a buffer containing 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM Na$_2$EDTA, 1 mM dithiothreitol, 9% glycerol (v/v). Fluorescence emission from the enzyme’s tryptophan residues was observed through a 320 nm long-pass filter. The instrument dead time was 1.38 ms. Typically, each trace shown is the average of at least five independent experiments. The concentration of Fpg protein in all experiments was 1.5 µM, and concentrations...
of oligodeoxyribonucleotide (ODN) substrates were varied in the range 0.25–4.0 μM. Corrections for bleaching of enzyme fluorescence and inner filter effect were as described (27).

**Kinetic data analysis**

The global non-linear least-squares fitting was performed using DynaFit software (BioKin Ltd) (29). Differential equations were written for each species in the mechanisms described by Schemes 1–3 (see Results), and the stopped-flow fluorescence traces were directly fit by expressing the corrected fluorescence intensity \( F_i \) at any reaction time \( t \) as the sum of the background fluorescence \( (F_b) \) and the fluorescence intensities of each protein species:

\[
F_c = F_b + \sum_{i=0}^{n} F_i(t)
\]

where \( F_i(t) = f_i[E_i(t)] \), \( f_i \) are coefficients of specific fluorescence for each discernible Fpg conformer, and \( [E_i(t)] \) are the concentrations of the conformers at any given time \( t \) (\( i = 0 \) relates to the free protein; \( i > 0 \), to the protein–DNA complexes). These specific fluorescence coefficients describe only the part of fluorescence that changes due to DNA binding.

**Titration of Fpg protein with oligodeoxyribonucleotides**

To determine the dissociation constant \( K_d \) of the complex of Fpg with the end product of the lesion excision, the enzyme was titrated with the mixture of oligonucleotides d(GGAAGGGCCGAGAG), d(CCTCTCp) and d(pCCTTCC) or with these oligonucleotides individually. The value of \( K_d \) was determined from equations 2–5:

\[
K_d = \frac{[Fpg] \cdot [ODN]}{[complex]}
\]

\[
[Fpg] = \frac{F - F_{\infty}}{F_0 - F_{\infty}} [Fpg]_0
\]

\[
[complex] = [Fpg]_0 - [Fpg]
\]

\[
[ODN] = [ODN]_0 - [complex]
\]

where, \( F_0 \), \( F \) and \( F_{\infty} \) are fluorescence intensities of Fpg without added oligonucleotide, at any given concentration of oligonucleotide, and at the saturating concentration of oligonucleotide, respectively. \([Fpg]_0 \) and \([ODN]_0 \) are the total concentrations of Fpg and oligonucleotide, \([Fpg] \) and \([ODN] \) are the concentrations of uncomplexed Fpg and oligonucleotide, [complex] is the concentration of the Fpg–oligonucleotide complex.

**RESULTS**

**Rationale**

In the previous study we have shown that the formation of a primary encounter complex between Fpg and 8-oxoG-containing substrate is followed by a stage of conformational adjustment of the enzyme and the substrate (27). The Fpg-dependent reaction therefore involves several steps: \( E + S \leftrightarrow ES \) (primary complex formation) \( \leftrightarrow ES^I \) (conformational adjustment of DNA and Fpg) \( \leftrightarrow ES^H \) (base excision and Schiff base formation) \( \leftrightarrow E-S^H \) (δ-elimination) \( \leftrightarrow E-S^IV \) (δ-elimination) \( \leftrightarrow ES^V \) (Schiff base hydrolysis) \( \leftrightarrow E + P \).

To improve our understanding of the identity of conformational changes responsible for substrate recognition and reaction chemistry, we designed a series of duplex dodecameric oligonucleotide Fpg substrates and ligands that contained either 8-oxoG, AP or F residues in the sixth position of one strand (Fig. 1). We have shown earlier (27) that an undamaged dodecamer is bound by Fpg with a single-phase fluorescence intensity change, which was attributed to the formation of an non-specific encounter complex. Going one step further, the F-containing substrate is specifically bound but not chemically processed by Fpg. In this case, the enzyme should only undergo conformational rearrangements related to the primary encounter and subsequent substrate recognition, but not to catalytic events. These catalytic events should be evident with AP- and 8-oxoG-containing substrates, the latter showing all catalytic steps and the former missing the base excision step. The series of substrates used in the present study thus allows specific attribution of every conformational change to a specific phase of Fpg-dependent catalysis. In addition, if a substrate contains 8-oxoG or AP, the product of excision of the deoxyribose residue of the damaged nucleotide, a duplex with one strand broken into a hexamer and a pentamer, should be at equilibrium with its individual oligonucleotide components. As a result, the fluorescence time-course traces may contain not only the segments resulting from complexation of Fpg with the substrate and from subsequent catalytic steps but also the segment indicative of the dissociation of the enzyme–product complex. To observe this stage, we analysed fluorescence traces obtained under single-turnover conditions, or with small excess of the substrate.

**Binding of Fpg to the F-containing duplex**

The fluorescence traces obtained for the non-cleavable F-containing ligand are presented in Figure 2. It is seen clearly that fluorescence intensities decrease with time, with higher substrate concentrations causing more pronounced decreases in fluorescence. The shapes of the curves and their analysis by global fitting suggest that the process of Fpg binding to this ligand proceeds through at least four phases (Scheme 1).

Bimolecular encounter and primary binding of the enzyme to DNA is observed over a 5–10 ms period, as has been described for the non-specific ligand containing a normal guanosine residue in place of the lesion within a sequence identical to that used here (27). This step corresponds to the formation of the (E-F)1 complex in Scheme 1. In the time period 20 ms to 1 s at least three additional stages were

\[
E + F \xrightleftharpoons{k_1^F} (E\cdot F)1 \xrightleftharpoons{k_2^F} (E\cdot F)2 \xrightleftharpoons{k_3^F} (E\cdot F)3 \xrightleftharpoons{k_4^F} (E\cdot F)4
\]

Scheme 1. Fpg binding to an F-containing duplex. F, F-containing ligand; (E-F)i, different enzyme–ligand complexes.
observed, none of which exists for the non-specific ligand (27). As mentioned above, Fpg induces DNA kinking and localized melting; the resulting significant structural changes may be key steps in adjustment of specific duplex DNA to the conformation optimal for catalysis. The ability of DNA to undergo this kind of distortion depends on several structural characteristics of DNA; such as roll, shift, twist or rise (30–32). Abasic sites in DNA are thermodynamically greatly destabilizing and increase DNA flexibility (33–35). It is therefore reasonable to suggest that, unlike an undamaged oligonucleotide duplex, the F-containing ligand can undergo drastic conformational changes on association with Fpg. The three additional stages (E´F)², (E´F)³ and (E´F)⁴ most likely mirror Fpg structural changes associated with DNA kinking, unstacking and insertion of the enzyme’s hydrophobic residues between the strands of the duplex. Since F does not form a Schiff base covalent intermediate with Fpg, its conformational adjustment in the enzyme’s active site is probably not fully identical to that of the true substrates. However, the F residue is very likely everted from DNA, as are other AP site analogues in Fpg–DNA complexes (20,21) and F in DNA complexed with human 8-oxoG–DNA glycosylase hOgg1 (36), making the F-containing ligand a good model to study the stages associated with the damaged nucleotide extrusion. Transition from F to a cleavable AP-containing substrate must be accompanied by an increase in the number of observed discernible fluorescence stages, which may be associated with additional conformational adjustment, substrate conversion or both.

**Binding and cleavage of AP-containing substrate by Fpg**

For the cleavable AP-containing substrate, in addition to the four steps found with F one more step is easily observed at the last segment of fluorescence traces, at times 10–100 s (Fig. 3). The fluorescence traces displayed clear minima, which were sharp when the initial concentration of the substrate (0.25, 0.5, 1.0 or 1.5 μM) did not exceed the total enzyme concentration (1.5 μM) but became flat with increase in substrate concentration (2, 3 or 4 μM), indicating the existence of a steady-state phase under these conditions. At lower substrate concentrations (0.25–1 μM) the intensity of Fpg fluorescence increases along the last segment of the trace and returns to the levels of fluorescence characteristic of the uncomplexed protein. With higher substrate concentrations the fluorescence intensity at the end of the experiment is below the starting fluorescence level (the higher the substrate concentrations, the lower the final fluorescence intensity). Therefore, a minimal mechanism
that can account for the observed fluorescence traces is described by Scheme 2.

The shapes of the fluorescence traces were identical for AP- and F-containing duplexes, with the exception of the last segment. In the case of the AP-containing substrate, we suggest that the stages 1–4, as with the non-cleavable F-containing ligand, reflect the formation of a primary complex and conformational adjustment during eversion of the damaged nucleotide. The last stage characterized by the rate constant $k_{5\text{AP}}$ is most likely associated with the chemical step, which leads to the excision of a modified ribose moiety and formation of two short oligonucleotides. In the case of the AP-containing substrate, removal of the abasic deoxyribose residue proceeds through two main catalytic steps, $\beta$- and $\delta$-elimination, with the formation of a covalent Schiff base intermediate (14,15). An increase in the fluorescence intensity in the final segment of the traces reflects the release of the enzyme from its complex with the product. The fifth reaction stage must therefore include release of the gapped product ($P_{\text{gap}}$) and its dissociation to a mixture of three individual oligonucleotides ($P_{\text{mix}}$). However, the last segment of fluorescence traces lacked fluorescence changes attributable to sub-stages of the catalytic process, and it was not clear which catalytic step ($\beta$- and $\delta$-eliminations, hydrolysis of the Schiff base or decomposition of the E·P complex) is rate limiting.

**Interactions of Fpg with the reaction product**

To estimate the dissociation constant for the enzyme–product complex, we titrated Fpg with a mixture of three oligonucleotides representing the incision product, and with each of these oligonucleotides individually. The short oligomers ($n = 5–6$) cannot form stable duplexes with the complementary 12mer strand under the conditions used in our experiments (37). However, many DNA-binding proteins are able to stabilize such short duplexes in the bound form (26); in particular, Fpg from *Bacillus stearothermophilus* was successfully crystallized as a complex with a short gapped end-product duplex (20). The change in Fpg protein fluorescence is shown in Figure 4. The triple oligonucleotide complex produced the same effect as did individual oligonucleotides at the same concentrations; this effect did not depend on oligonucleotide sequence or structure. Thus, the protein formed non-specific complexes with all these oligonucleotides, with the calculated dissociation constant $K_d = 5.3 \, \mu M$. This observation is in good agreement with reported microcalorimetric measurements of Fpg binding to the gapped product, which showed much lower affinity of the enzyme to the product than to an F-containing duplex (38). Since the affinity of Fpg for the product is essentially the same as for the individual oligonucleotide strands, the rates of association and dissociation of the product at least should not be less than that for the undamaged oligonucleotide duplex (27), and product dissociation should not be rate limiting during the last segment of fluorescence traces observed with the AP-containing duplex.

**Binding and cleavage of 8-oxoG-containing substrate by Fpg**

Compared with the AP-containing substrate, processing of the 8-oxoG-containing substrate took more time (Fig. 5). When comparing data for AP- and 8-oxoG-containing substrates it should be taken into account that adjustment of their structure and respective conformational changes in the Fpg molecule may be different in some respects due to additional bonds formed between the protein and the 8-oxoG base (25). Formation of all necessary contacts between Fpg and an 8-oxoG-containing substrate might be significantly slower than the same process involving an AP-containing substrate, which forms fewer contacts with the enzyme’s active site. Indeed, additional broad peaks were observed in the time period 0.5–5 s with the 8-oxoG-containing substrate and the minima at $>10 \, s$ became wider compared with the AP-containing substrate. The last segment of fluorescence traces, where an increase in fluorescence is observed, occurred at times 4- to 5-fold longer than with AP-containing substrate. Clearly, in the case of the 8-oxoG-containing substrate these features of fluorescence traces reflect the glycosylase reaction. To be
assisted catalysis during 8-oxoG excision by hOgg1 (39).

Verdine and colleagues reported a unique case of product-the presence of an 8-oxoG residue in the substrate. Recently, remained unclear whether conformation of Fpg depends on common intermediate (E´AP)4 (Scheme 4). However, it substrates should be a superposition of Schemes 2 and 3 with a obtained for all three duplexes are presented in Table 1.

Table 1. Rate constants for interaction of Fpg protein with F-, AP- and 8-oxoG-containing substrates

<table>
<thead>
<tr>
<th>Rate constants</th>
<th>Substrate or ligand X</th>
<th>F</th>
<th>AP</th>
<th>OG</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{X,s}^{N}$, M⁻¹s⁻¹</td>
<td>2.3 $\times 10^{10}$</td>
<td>1.5 $\times 10^{8}$</td>
<td>8.0 $\times 10^{8}$</td>
<td>3.2 $\times 10^{8}$</td>
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<tr>
<td>$k_{X,s}^{s}$, s⁻¹</td>
<td>2.7 $\times 10^{9}$</td>
<td>270</td>
<td>250</td>
<td>890</td>
</tr>
<tr>
<td>$k_{X,s}^{s}$, s⁻¹</td>
<td>6.0</td>
<td>36</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>$k_{X,s}^{s}$, s⁻¹</td>
<td>0.02</td>
<td>65</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>$k_{X,s}^{s}$, s⁻¹</td>
<td>10.0</td>
<td>10</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>$k_{X,s}^{s}$, s⁻¹</td>
<td>0.6</td>
<td>40</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>$k_{X,s}^{s}$, s⁻¹</td>
<td>0.04</td>
<td>11</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>$k_{X,s}^{s}$, s⁻¹</td>
<td>0.01</td>
<td>1.0</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>$k_{X,s}^{s}$, s⁻¹</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
<td></td>
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<tr>
<td>$k_{X,s}^{s}$, s⁻¹</td>
<td>0.03</td>
<td></td>
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<tr>
<td>$k_{X,s}^{s}$, s⁻¹</td>
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<td></td>
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<tr>
<td>$k_{p}^{p} \mu M$</td>
<td>1.8</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mean values; the standard error is within 30%. The standard error increases with increasing stage number. The error for $k_{p}$ is within 80%. OG, 8-oxoG.

*From (27).*

*Compared with the value for $k_{p} = 3.7 \mu M$ reported earlier for 8-oxoG (27) and $k_{p} = 5.3 \mu M$ obtained from titration experiments.

excised, the 8-oxoG base must be first extruded from DNA helix and inserted into the active site pocket of the enzyme, followed by hydrolysis of the N-glycosidic bond with the release of the damaged base. The step at 0.5–5 s most probably corresponds to the eversion of 8-oxoG followed by glycosidic bond hydrolysis. The shift of the last segment of the traces, where the fluorescence increases, towards longer times indicates that the glycosylase reaction is the rate-limiting step in the whole process of 8-oxoG excision, confirming predictions made from steady-state kinetic experiments (10).

Therefore, the N-glycosidic bond hydrolysis may be added to the reactions attributed to this segment of the traces for the AP-containing substrate (Schiff base formation, β- and δ-elimination, Schiff base hydrolysis and product release).

Analysis of parts of the fluorescence traces before the last minimum showed that this part of the reaction might be described by a scheme including five equilibria. Accordingly, Scheme 3 illustrates the full reaction process. Here, E-OG1 is the encounter complex, and E-OG2–E-OG5 are conformers of the specific enzyme–substrate complex formed on recognition of 8-oxoG in the active site of enzyme. The step characterized by rate constant $k_{OG}$ corresponds to all chemical steps (except, possibly, base excision) and to the release of enzyme from the complex with substrate.

Equilibrium stages reflect the steps of mutual adjustment of the structures of Fpg and its substrate towards the catalytically optimal conformation. Hence, recognition of substrates by Fpg is a multistage process, taking up to 10 s. It is followed by catalytic steps, complete after 50–200 s under the conditions used in our experiments. A summary of the kinetic data obtained for all three duplexes are presented in Table 1.

The full kinetic scheme describing Fpg action on different substrates should be a superposition of Schemes 2 and 3 with a common intermediate (E·AP)4 (Scheme 4). However, it remained unclear whether conformation of Fpg depends on the presence of an 8-oxoG residue in the substrate. Recently, Verdine and colleagues reported a unique case of product-assisted catalysis during 8-oxoG excision by hOgg1 (39). Existing in the anionic form immediately after the breakage of the N-glycosidic bond, 8-oxoG was shown to be a cofactor in β-elimination reaction, with N9 of 8-oxoG abstracting a C2′ proton of the deoxyribose moiety. Since the 8-oxoG base is very poorly soluble, 8-bromoguanine (8-BrG) was used as its analogue for kinetic analysis of hOgg1 (39). We investigated the effect of 8-BrG on the fluorescence traces produced during the cleavage of the AP- and 8-oxoG-containing substrates by Fpg. No influence of 8-BrG on the traces was observed, indicating that the excised 8-oxoG base is not a cofactor in the reactions catalysed by Fpg. This is perhaps not surprising in view of the lack of sequence or structural similarity between hOgg1 and Fpg (19,40).

**DISCUSSION**

**Structural basis for Fpg fluorescence**

Accurate attribution of fluorescently discernible steps of the Fpg-catalysed reaction to conformational changes in the enzyme requires information about possible effects of the conformational changes on the tryptophan residues responsible for intrinsic protein fluorescence. The structure of *E.coli* Fpg complexed with DNA (19) contains five tryptophan residues (Fig. 6). Three of these, Trp66, Trp113 and Trp115, are found buried or half-buried in the quite rigid N-terminal β-sandwich core domain and are unlikely to be the major moieties that change their fluorescence upon DNA binding (Trp66, which is partly exposed to the solution, may still contribute to the observed effect). Trp34 and Trp156 are much better candidates for generating a fluorescence signal dependent upon the protein conformation. Trp34 lies in the N-terminal domain, exposed mostly into solution and quite close to a flexible hinge linking the N- and C-terminal domains of the protein. Since the DNA-binding cleft lies between these domains, the linker may change its conformation upon DNA binding. In addition, C=O atom of Trp34 makes a van der Waals contact with C5′ of the third nucleotide 3′ to the lesion. Another tryptophan residue, Trp156, lies in the C-terminal domain of the protein in the H2TH motif, which is crucial for DNA binding. Trp156 is sandwiched between Lys149 and...
uncomplexed Fpg (18) reveals significant changes in philus covalently complexed with DNA (19) and Thermus thermo-binding, such as disruption of the bonds formed by N potential hydrogen bonding patterns of Asn168 upon DNA E.coli binding. A comparison of the structures of motif may also undergo conformational changes upon DNA conserved Asn168 residue in the H2TH motif. The H2TH Leu162, being a part of an extensive packing structure, which is responsible for correctly orienting the critical, absolutely conserved Asn168 residue in the H2TH motif. The H2TH motif may also undergo conformational changes upon DNA binding. A comparison of the structures of E.coli Fpg covalently complexed with DNA (19) and Thermus thermophilus uncomplexed Fpg (18) reveals significant changes in potential hydrogen bonding patterns of Asn168 upon DNA binding, such as disruption of the bonds formed by Ne2 and the amido nitrogen of Asn168 with other protein moieties, and their replacement by bonds to the damaged nucleotide. Such changes could affect the conformation of the H2TH domain and, consequently, the environment of Trp156 strongly enough for a change in fluorescence to occur. Therefore, the observed fluorescence traces most likely reflect conformational changes in the linker domain and the H2TH domain. Other changes, which may occur in functionally important parts of the protein lacking tryptophan residues (such as the α1 helix where the catalytic proline is found, or the C-terminal zinc finger), may not be detected by our assay.

**Figure 6.** Tryptophan residues in the structure of Fpg. A cartoon representation of the protein is shown with DNA in yellow sticks. Side chains of tryptophan residues are shown in blue sticks and labelled.

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**Kinetic model attribution**

According to the almost universally accepted model of action of DNA glycosylases, the enzyme first binds DNA non-specifically and slides along it in the process of passive one-dimensional diffusion (41). When the enzyme encounters a lesion, it recognizes either certain specific determinants, or a local change in DNA thermodynamic parameters, e.g. the energy of DNA bending (42). For catalysis to occur, the damaged nucleotide must be everted from DNA to expose its C1’ to the catalytic machinery of the enzyme (22). Subsequent catalytic events depend on the particular glycosylase and may involve protonation of various atoms of the lesion, attack at C1’ of the lesion by an activated water molecule or an enzyme’s nucleophilic group, formation of a covalent reaction intermediate, and β- and δ-elimination steps. New protein–DNA (or within-DNA) interaction elements formed upon nucleotide eversion act as final recognition mechanisms, greatly amplified by requirements for highly precise alignment of electron orbitals for efficient catalysis (26,43,44). This second recognition step is most probably required to prevent erroneous excision of normal DNA bases adventitiously recognized by the enzyme.

We intentionally designed our substrates to be short in order to assist product release. Indeed, under single-turnover conditions the fluorescent traces displayed quite sharp minima. The fluorescence initially decreased, the shape of the traces being indicative of a multistage process. Later the fluorescence intensity increased for the cleavable substrates in a process described by a single kinetic step. When substrate concentrations exceeded that of the enzyme, the minima broadened, indicative of the steady-state stage of the reaction.

We did not observe any early changes in fluorescence that might be attributed to the one-dimensional enzyme diffusion. Since the substrates were only slightly longer than the enzyme footprint (19,25), the contribution of one-dimensional enzyme sliding towards the site of damage should be negligible. The enzyme most probably associated directly with the damaged unit; however, this complex is not yet truly specific, since the damage recognition step has not occurred yet (see below). For all ligands, this complex formed at very similar rates, characterized by the rate constant $k_1$, which varied by no more than an order of magnitude (Table 1). However, the rate of dissociation of the primary encounter complex varied significantly (Table 1), being the highest for undamaged duplex, and dropping 3-fold for the 8-oxoG-containing substrate and 10-fold for F- and AP-containing substrates. The calculated equilibrium constants for the first stage are $0.85 \times 10^5$, $5.55 \times 10^5$, $3.2 \times 10^6$ and $3.6 \times 10^5$ M$^{-1}$ for undamaged, F-, AP- and 8-oxoG-containing substrate, respectively. Therefore, Fpg displayed the highest affinity to the AP-containing substrate, and the lowest affinity to undamaged DNA, paralleling the preferences found in the microcalorimetric titration experiments (38).

Four fluorescence-trace phases observed in the case of the uncleavable F substrate clearly represent pre-catalytic steps up to damaged nucleotide eversion. Although no data are available on the structure of F complexed with Fpg, there is no reason to believe that such ligand will bind Fpg without the eversion. All solved structures of DNA glycosylases complexed with abasic site analogues (20,21,36,45–47) show the damaged nucleotide everted. In addition, the high affinity of Fpg for F (10) also suggests that the pre-catalytic, rather than the non-specific complex is formed with this lesion. The pre-catalytic complex probably differs significantly from the non-specific complex, as suggested by the sharp kink in DNA found in the former (19–21). This kink moves the substrate DNA away from the protein–DNA interface, so that approximately half of the DNA-binding cleft remains unoccupied.
when specific DNA is bound. DNA binding, its gross rearrangement, and nucleotide eversion will very likely be accompanied by conformational changes in the linker hinge and the H2TH motif. Thus, (E-F)1 likely represents an ensemble of Fpg molecules bound to DNA non-specifically, (E-F)2, the initial recognition complex with the F lesion, (E-F)3 is the pre-catalytic complex with F ejected out of the DNA helix into the Fpg active site. Formation of (E-F)4 may represent an isomerization step (insertion of protein residues into DNA helix) preventing ejected nucleoside returning into DNA helix, similarly to the reaction mechanism proposed for E.coli uracil-DNA glycosylase and E.coli MutY (48,49). Naturally, all these steps are reversible.

When a cleavable AP substrate is presented to the enzyme, an additional, irreversible step is observed. The catalytic events following nucleotide eversion by Fpg may be divided into in four stages: formation of a Schiff base intermediate, β-elimination, δ-elimination and hydrolysis of the Schiff base intermediate. It is not known to what extent these events change the enzyme’s conformation and why a well-defined single fluorescence event is observed. The structural data suggest that β- and δ-elimination may involve the residues of the N-terminal domain and the zinc finger motif, but not the H2TH motif (19–21). In B.stearothermophilus Fpg, conformations of the protein are virtually identical in a non-covalent complex with a reduced AP site analogue (a pre-catalytic stage), a covalent borohydride-trapped complex (Schiff base stage preceding β-elimination), and a non-covalent complex with gapped DNA (post-incision complex) (20). This may indicate that the catalytic steps, when the conformations of DNA and Fpg have been mutually adjusted, do not require much gross movement but occur mostly through electron and proton transfer in the active centre. Thus, it is reasonable to propose that chemical steps of Fpg catalysis occur mostly with little change in fluorescence, and only one of them is actually observed in our experiments as a separate fluorescence event.

For many DNA glycosylases, product release is rate limiting (50–52). Rabow et al. observed a burst phase during the time course of cleavage of an AP-containing substrate by wild-type Fpg but not the Fpg K155A mutant, and suggested that product release is also rate limiting for Fpg (53). We, in an apparent disagreement, have estimated the rate of product release to be of the same order of magnitude as the rate of release of undamaged duplexes. It should be noted, however, that in the Fpg system with more than two catalytic steps, burst rate kinetic with gel electrophoresis detection would reflect the existence of a slow step after first incision, and not necessarily slow product release, whereas we measured product release per se, after all catalytic steps were completed. Thus, δ-elimination or Schiff base hydrolysis may be the rate-limiting step for Fpg acting on abasic sites.

The change in fluorescence intensity observed for the 0.1–1 s segment is notably less for the AP-containing substrate than for the F-containing substrate, although the shape of traces is very similar. Therefore, adjustment of the enzyme conformation is somewhat different for these two ligands.

Finally, when the substrate contained 8-oxoG, one additional fluorescence step was observed before the catalytic AP lyase events. This is evident from significant lengthening of the fluorescence peak ~100 ms into the range of seconds. Another characteristic feature is the shift of the minima at the end of fluorescence traces towards longer times. These features are naturally attributable to the DNA glycosylase reaction events. Obviously, eversion of the 8-oxoG nucleotide requires more extensive adjustment of the enzyme conformation compared with the AP flip-out. As with the abasic substrate, in the case of the 8-oxoG-containing substrate, all catalytic stages are not seen as fluorescently discernible steps.

In earlier work (27), we made several suggestions regarding the nature of conformational changes during Fpg interaction with an 8-oxoG-containing substrate. The present, more detailed analysis of fluorescent traces resulting from interactions of Fpg with substrates or ligands of different kinds has allowed a better estimate of the number of conformational changes and the nature of these changes. Stages 1–3 for the 8-oxoG-containing substrate are identical to the same stages for the AP-containing substrate, whereas conformational changes at stage 4 are different. After an increase in the enzyme fluorescence at stage 3, it decreased at stage 4 with the AP-containing substrate; a sharp peak was observed in the fluorescence traces. Such a decrease is not observed until stage 5 with the 8-oxoG-containing substrate, where fluorescence at stage 4 did not change appreciably, resulting in a shallow peak corresponding to stages 3 and 4. Therefore, stage 5 also has different mechanistic meaning for AP- and 8-oxoG-containing substrates: for the former, stage 5 corresponds to DNA strand

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Scheme 4. Binding and cleavage of any substrate by Fpg.
cleavage and product release, and for the latter, to the transition to an immediate pre-catalytic conformation; catalytic steps and product release corresponds to stage 6 in the case of 8-oxoG. Scheme 4 summarizes the kinetic models for Fpg, representing the combination of models obtained for AP- and 8-oxoG-containing substrates. Complex (E-AP)4 is further converted into a Schiff base intermediate, which is then processed to an enzyme–product complex. It is reasonable to conclude that the (E-AP)4 complex is directly produced from a pre-formed catalytically competent enzyme–substrate complex (E-OG)5. The structure of (E-AP)4 probably does not depend on whether the process starts with 8-oxoG or AP, as indicated by the insensitivity of the reaction traces to 8-BrG.

The conversion of (E-OG)5 into (E-AP)4 corresponding to the glycosidic reaction represents the rate-limiting step of the process. This reaction is characterized with the kinetic constant 0.04 s−1 (k_5^{AP}), Scheme 2) and proceeds faster than the cleavage of glycosidic bond.

In summary, our data indicate that the initial phases of the fluorescence traces, where the most pronounced changes in fluorescence occur, reflect the stages of Fpg binding to DNA, lesion recognition and damaged nucleotide evasion, with the mutual adjustment of DNA and enzyme structure into a catalytically competent conformation. The conversion of (E-OG)5 into (E-AP)4 and then to E-P, involving β- and δ-elimination steps and Schiff base hydrolysis, are not observed as separate stages from intrinsic tryptophan fluorescence. Rather they are observed as a single stage characterized by a constant of a rate-limiting step. In the case of 8-oxoG, the rate-limiting step is most probably the hydrolysis of the N-glycosidic bond, since the characteristic times for cleavage of 8-oxoG and AP are quite different.

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