Conformational Dynamics of DNA Repair by *Escherichia coli* Endonuclease III*

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Conformational dynamics of *Escherichia coli* endonuclease III (Endo III or Nth) is a DNA glycosylase with a broad substrate specificity for oxidized or reduced pyrimidine bases. Endo III possesses two types of activities: N-glycosylase (hydrolysis of the N-glycosidic bond) and AP lyase (elimination of the 3′-phosphate of the AP-site). We report a pre-steady-state kinetic analysis of structural rearrangements of the DNA substrates and uncleavable ligands during their interaction with Endo III. Oligonucleotide duplexes containing 5,6-dihydrouracil, a natural abasic site, its tetrahydrofuran analogs 2-aminopurine and 1,3-diaza-2-oxophenoxazine as environment-sensitive reporter groups. The results suggest that Endo III induces several fast sequential conformational changes in DNA during binding, lesion recognition, and adjustment to a catalytically competent conformation. A comparison of two fluorophores allowed us to distinguish between the events occurring in the damaged and undamaged DNA strand. Combining our data with the available structures of Endo III, we conclude that this glycosylase uses a multistep mechanism of damage recognition, which likely involves Gln^41 and Leu^81 as DNA lesion sensors.

Base modifications induced in DNA by a number of endogenous or exogenous factors lead to adverse effects in cells (1–3). Some damaged DNA bases are potentially mutagenic, and are involved in carcinogenesis and aging (4). Other lesions, such as thymine glycol and 5,6-dihydrouracil (dHU),^3 can block DNA polymerase during the replication (5–7). Non-bulky small base lesions, derived from deamination, alkylation, or oxidation, are excised and replaced in the base excision repair pathway. The first enzymatic step in the base excision repair pathway is typically the excision of the substrate base from duplex DNA by a DNA glycosylase, one of the enzymes that catalyze the cleavage of the N-glycosidic bond between the substrate base and the 2′-deoxyribose, creating an abasic (AP) site.

In *Escherichia coli*, there are eight known DNA glycosylases (8). The enzyme endonuclease III (Endo III) mainly excises pyrimidine-derived lesions such as thymine glycol, uracil glycol, dHU, 5,6-dihydrothymine, 5-hydroxy-5,6-dihydrothymine, 5-hydroxy-5,6-dihydrouracil, 5-hydroxyuracil, 5-hydroxycytosine, alloxaan, urea, 6-hydroxy-5-hydouracil, 5,6-dihydroxyuracil, 6-hydroxy-5-hydroxycytosine, and products of fragmentation of these lesions (9–11).

Endo III is a bifunctional DNA glycosylase possessing N-glycosylase and AP lyase activities (Fig. 1) (12, 13). The principal amino acids involved in the catalysis are Lys^120 and Asp^138. The former is the nucleophile that attacks the C1’ atom of deoxyribose, resulting in the cleavage of the N-glycosidic bond and subsequent formation of a Schiff base covalent intermediate (Fig. 1, *step 1*). The following β-elimination reaction leads to the departure of the 3′-phosphate. The subsequent Schiff base hydrolysis releases the enzyme and leads to formation of a single-strand break in DNA duplex with an α,β-unsaturated aldehyde at the 3’-end and a phosphate at the 5’-end (Fig. 1, *step 2*).

The x-ray structures of free Endo III from *E. coli* as well as three complexes of Endo III from *Geobacillus stearothermophilus* with DNA have been published (14, 15). The overall structure of enzyme shows two similarly sized globular domains separated by a deep DNA-binding groove that contains the active site cavity (Fig. 2). Endo III binding induces gross confor-
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Although the three-dimensional structure of the Endo III-DNA complex is established, it tells us little about the mechanism of substrate recognition by this enzyme, because the DNA in the structures contains no damaged base. The overall mode of interaction of Endo III with DNA is very similar to that seen with other members of the HhH-GPD structural superfamily, making it possible to compare the kinetic and conformational features of DNA binding and damage recognition with other well studied members of this superfamily (16). Our previous studies (17–21) of another member of the helix-hairpin-helix (HhH)-GPD superfamily, human 8-oxoguanine-DNA glycosylase (hOGG1), revealed that hOGG1 use multistage mechanism of lesion recognition that includes step-by-step involvement of different amino acids. It was concluded (20) that at the initial step of DNA binding Arg154 and Arg204 (corresponding to Arg78 and Arg84 of G. stearothermophilus Endo III, Fig. 3B) “pull out” the cytosine base located opposite the oxoG base and Tyr203 (spatially corresponding to Leu82 in G. stearothermophilus Endo III, Fig. 3B) is partially inserted into the DNA duplex. These interactions lead to DNA distortion and eversion of the oxoG base from DNA to the intermediate base-binding site (exo-site) of the enzyme. A thermodynamic analysis (21) argues that the initial step of the DNA substrate binding is mainly governed by a negative enthalpy change due to the formation of favorable contacts between hOGG1 and DNA, as well as due to the partial desolvation of the interface resulting in positive entropy change. The oxoG base is then fully placed into the active site and forms specific interactions within the base recognition pocket detected in the structure of the pre-catalytic hOGG1-DNA complex (22). In addition, Asn149 (corresponding to Gln42 in G. stearothermophilus Endo III, Fig. 3B) forms a hydrogen bond between the amide carbonyl of its side chain and the amino group of the opposite cytosine. Interestingly, hOGG1 can also guide the undamaged G base through the exo-site into the active site, albeit less efficiently, but cannot cleave its N-glycosidic bond indicating strong discrimination selectivity between damaged and undamaged DNA (23, 24). A stepwise thermodynamic analysis (21) reveals that the discrimination of nonspecific G base versus specific oxoG base mostly occurs at the second step of the DNA binding. At the last pre-catalytic step the enzyme-substrate complex is finally adjusted to a catalytically competent conformation. This process is characterized by large endothermicity compensated by a significant increase in entropy likely originating from dehydration of the DNA grooves.

Here we present a kinetic analysis of conformational changes in DNA with another DNA glycosylase from the HhH-GPD structural superfamily, E. coli Endo III. Two fluorescent reporters, 2-aminopurine (aPu) placed 3' to the damaged nucleotide and tCO placed opposite to the lesion were chosen for detection of DNA dynamics. Dodecamer oligodeoxyribonucleotide duplexes contained centrally placed specific lesions: dHU, the natural AP site and its uncleavable analogue (3-hydroxytetrahydrofuran-2-yl)methyl phosphate (F) or undamaged nucleotide. The substrates containing dHU are subject to the full enzymatic cycle, which includes DNA binding, N-glycosidic bond cleavage, β-elimination, and product release. The substrates containing an AP site are limited to β-elimination and product release. The undamaged DNA duplexes and duplexes contain-
Experimental Procedures

**Purification of Endo III**—To purify recombinant *E. coli* Endo III, 1 liter of *E. coli* JM105 carrying the pNth 10 (25, 26) plasmid were grown in 2× YT broth supplemented with 50 µg/ml of ampicillin at 37 °C until A600 = 0.6–0.8, then shifted to 30 °C and induced overnight with 0.1 mM isopropyl β-D-1-thiogalactopyranoside. The cells were harvested by centrifugation, resuspended in 30 ml of the lysis buffer (20 mM HEPES-KOH, pH 7.8, 40 mM NaCl), and lysed using a French press. Insoluble material was pelleted by centrifugation. The supernatant was passed through a Q-Sepharose column (GE Healthcare, Little Chalfont, UK) in the same buffer but containing 250 mM NaCl. The eluate was diluted 2-fold by 20 mM HEPES-KOH (pH 7.8) and loaded into a heparin column (GE Healthcare). Endo III was eluted using a 100–1500 mM NaCl gradient in 20 mM HEPES-KOH. The fractions containing Endo III were collected, glycerol was added to 50%, and the samples were stored at −20 °C.

**Oligodeoxynucleotides**—The sequences of oligodeoxynucleotides used in this work are listed in Table 1. The oligodeoxynucleotides were synthesized by the standard phosphoramidite method using an ASM-700 synthesizer (BIOSSET, Novosibirsk, Russia) in the ICBFM Laboratory of Bionanotechnology. The phosphoramidites were purchased from Glen Research (Sterling, VA). Duplexes were prepared by annealing the modified and the complementary strand at a 1:1 molar ratio.

**Stopped-flow Experiments**—The experiments were conducted essentially as described previously (27–29). All experiments were carried out at 25 °C in buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 7% glycerol (v/v). An SX.18MV stopped-flow spectrometer (Applied Photophysics, Leatherhead, UK) fitted with a 150-W Xe arc lamp was used. The dead time of the instrument was 1.4 ms. The excitation wavelengths were 310 and 360 nm for the aPu and tCO fluorescent dyes, respectively. The emission was monitored using long pass wavelength filters (Corion) at 370 nm for aPu and 395 nm for tCO. Endo III was placed in one syringe of instrument and rapidly mixed with the substrate in another syringe. The concentration of substrates in all experiments was 1.0 µM, and the concentration of Endo III was varied in the 0.5–4.0 µM range. The reported concentrations of reagents are those in the reaction chamber after mixing. Typically, each trace shown is the average of four or more individual experiments; the reported rate constants represent the mean ± S.D. of such data sets. In the figures, the individual traces are manually offset for clarity.

**Kinetic Data Analysis**—The kinetic parameters were obtained by global non-linear fitting using the DynaFit software (BioKin, Pullman, WA) (30) as described previously (21, 31–33). The approach is based on the fluorescence intensity variation in the course of the reaction due to sequential formation and further transformation of the DNA-enzyme complex and its conformers. The software performs numerical integration of a system of differential equations with subsequent non-linear least-squares regression analysis. The response factors of the intermediates (that are essentially the products of 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 the specific signal contributions. 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.
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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 was used. The fits of all relevant rate constants for the forward and reverse reactions, as well as the specific molar responses for all intermediate complexes, were optimized. After that rate constants for the forward and reverse reactions were fixed and additional optimization of the specific molar responses of intermediate complexes for individual kinetic traces was done.

Product Analysis—To analyze the products formed by Endo III, the substrates were 5'-32P labeled using phage T4 polynucleotide kinase and [γ-32P]ATP and the reaction was performed under the conditions described above. The products were precipitated by adding 10 volumes of 2% LiClO4 in acetone. The precipitates were washed three times with 100 μl of acetone, dried, dissolved in 4 μl of water and 3 μl of loading buffer (80% formamide, 20 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue), and analyzed by 20% denaturing PAGE. To observe formation of the AP site after base excision, the reaction mixture was treated with 0.3 M NaOH and heated for 15 min at 75 °C to cleave the DNA backbone at AP sites. The gels were visualized using Agfa CP-BU x-ray film (Agfa-GeaVert, Mortsel, Belgium), and the autoradiograms were scanned and quantified using Gel-Pro Analyzer software (Media Cybernetics, Rockville, MD).

The time course product accumulation were fitted by a single exponential function using Origin software (OriginLab Corporation, Northampton, MA) (Equation 1),

\[ \text{[Product]} = A \times [1 - \exp(-k_{obs}t)] \]  

where \( A \) is the amplitude, \( k_{obs} \) is the rate constant, and \( t \) is the reaction time.

Results

Conformational Transitions in the Damaged DNA Strand—The most informative approach to follow conformational transitions occurring in protein-DNA interactions through fluorescence changes involves a combination of the intrinsic protein fluorescence from tryptophan residues and DNA fluorescence from incorporated reporters. However, we were unable to detect changes in Endo III fluorescence in the reactions with any ligand or substrate, which is most likely due to suboptimal location of Trp residues or lack of significant conformation mobility in the protein molecule. Thus we have resorted to a set of fluorescent reporters to follow DNA dynamics in the complex.

The fluorescence analogous of DNA bases aPu (20, 34–36), 3-[β-D-2-ribofuranosyl]-6-methylpyrrolo[2,3-d]pyrimidin-2(3H)-one (Cpy) (31, 33, 37, 38), 3-hydroxycromone (3HC) (33, 39, 40), and 1,3-diaza-2-oxophenazine (tCO) (41–43) were incorporated in DNA substrates as reporter groups. Of these, aPu has been widely used as a reporter to study conformational transitions in DNA (27, 35, 44, 45). The fluorescence signal of aPu is strongly influenced by its surroundings; particularly, the fluorescence quantum yield of aPu is high in the polar environment and is considerably reduced when aPu is incorporated into nucleic acids (46). As a base in DNA, aPu does not disturb base stacking and forms an unusually stable base pair with cytosine (47).

We have first examined the activity of Endo III on DNA substrates containing aPu or other fluorescent reporters either 5' or 3' to dHU. The presence of aPu did not affect the reaction rate in comparison with the dHU substrate carrying no reporter (data not shown). The APu residue located immediately 5' to lesions was less sensitive to the conformational changes of DNA than when placed at the 3'-side (data not shown). In contrast, analysis of the product accumulation by PAGE showed that other fluorescent base analogs (Cpy, tCO, and 3HC) placed at the 5'-side of dHU make the substrate totally resistant to cleavage by Endo III (data not shown), similar to recent findings for DNA glycosylase Nei, which also excised oxidized pyrimidines but belongs to another structural superfamily (33). Therefore, we constructed 12-base pair oligodeoxyribonucleotide duplexes with an APu reporter on the 3'-side of the site of unmodified G, or modified F, AP, and dHU residues as specific ligands and substrates for Endo III (Table 1). The substrates containing dHU are subject to the full enzymatic cycle, which includes DNA binding, N-glycosidic bond cleavage, β-elimination, and product release. The substrates containing an AP site are limited to β-elimination and product release. The unmodified DNA duplexes and duplexes containing F reveal the conformational changes in DNA during binding with Endo III uncomplicated by catalytic steps.

G-aPu Ligand—The binding of undamaged G-aPu ligand by Endo III did not lead to appreciable changes in the aPu fluorescence intensity (data not shown) at any enzymesubstrate ratio indicating that this strand may be only slightly distorted in the complex of Endo III with nontarget DNA.

F-aPu Ligand—When the concentration of Endo III varied in the 0.5–4 μM range with the constant 1 μM concentration of the uncleavable analog of AP site, F-aPu ligand, the fluorescence traces indicated that the binding was essentially complete within 10 s (Fig. 4A). The amplitude of the fluorescence change was dependent on the Endo III concentration. The decrease in the aPu fluorescence intensity is suggestive of the transition of the aPu base into a more hydrophobic environment, most reasonably explained by an insertion of amino acid side chains of Endo III (such as Gln41) into the vacant space present in the DNA double helix due to the abasic void. As expected, the amplitude of aPu fluorescence intensity change increases with the increase in the Endo III concentration, reflecting a shift of binding equilibrium toward the complex. The kinetic data are satisfactorily described by Scheme 1 with a single equilibrium step. Rate and equilibrium constants derived from Scheme 1 are shown in Table 2.

AP-aPu Substrate—AP site is a cleavable substrate for Endo III, undergoing β-elimination after binding to the enzyme (see Fig. 1). Fig. 4B shows the time course fluorescence traces upon Endo III interaction with the AP-aPu substrate. The concentration of Endo III varied in the 1–4 μM range at the fixed 1 μM concentration of the substrate. The shapes of the fluorescence curves reveal four phases in the process: (i) a slight increase in fluorescence up to 0.2 s, (ii) a decrease phase up to 3 s, (iii) a final increase in the fluorescence intensity, and (iv) a plateau. The minimal kinetic mechanism corresponding to these fluores-
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TABLE 1
DNA substrates used in this study

| Shorthand | Sequence |
|-----------|----------|
| X-aPu     | C T C T C X aPu C T C T C |
| X = G, F, AP, dHU | G A G A G G C G A A G G |
| X/tC⁰    | C T C T C X C C T T C C |
| X = G, F, AP, dHU | G A G A G tC⁰ G G A A G G |
| X/N      | C T C T C X C C T T C C |
| X = AP, dHU | G A G A G N G G A A G G |

ence changes contains two reversible binding steps, one irreversible step of catalysis, and an equilibrium stage of the product release from the complex with the enzyme (Scheme 2). The rate and equilibrium constants obtained by fluorescence trace fitting are shown in Table 2. The affinity of Endo III for the natural AP site was ~12-fold higher than for its synthetic analog, F; the association constant $K_{bind}$ were $5 \times 10^6$ M$^{-1}$ and $0.4 \times 10^6$ M$^{-1}$, respectively. Moreover, the first binding step was characterized with a higher rate constant of complex formation ($k_1$) for the AP-aPu substrate than for the F-aPu ligand. Despite this, similar association constants $K_1$ were observed for both DNA duplexes. The observed difference in the affinity of Endo III for F- and AP-containing DNA is provided by the second binding step, absent in the F-aPu ligand. It can be inferred that the adjustment of the enzyme-substrate complex into a catalytically competent conformation requires the second step of the productive binding process. The third step in Scheme 2 is irreversible, most likely attributed to the chemical step of catalysis, i.e., AP lyase reaction, characterized with $k_3^{aPu} = 0.38 \pm 0.02$ s$^{-1}$. This rate constant is an order of magnitude higher than the rate constant for the same reaction characterizing hOGG1 with its notably low turnover rate (18).

dHU-aPu Substrate—Although no structure of Endo III in a complex with base-containing DNA is available, the structures of G. stearothermophilus Endo III bound to abasic DNA (15) suggests that the damaged base should be flipped out from the stack into the active site pocket. The eversion of dHU base from the DNA helix should lower the hydrophobicity of the environment of the aPu residue and therefore induce an increase in the aPu fluorescence signal. The observed fast increase in the aPu fluorescence intensity up to 5 ms (Fig. 4C) shows that Endo III induces the damaged base eversion at an early stage of DNA binding. After the fast eversion of the dHU base from DNA the void is plugged by amino acid side chains. The void-filling process, observed also with abasic DNA, is characterized by a decrease in the fluorescence intensity and extends up to 20 s. A further increase in fluorescence intensity may be associated with the catalytic step and release of the product from the active site of the enzyme. The minimal four-step kinetic scheme describing the observed changes of aPu fluorescence intensity is identical to that proposed for the AP-aPu substrate (Scheme 2) and contains two equilibrium steps of substrate binding followed by one irreversible chemical step and then by an equilibrium step of product release. The kinetic constants that satisfy Scheme 2 are presented in Table 2. As can be seen from their values, Endo III possesses ~4-fold lower affinity for dHU base than for AP site at the first binding step, whereas the second binding step, which corresponds to the void-filling process,
does not discriminate between AP and dHU substrates. The products of conversion of both specific substrate conversions have lower affinities for Endo III than AP and dHU substrates (compare $K_{\text{P}}^\text{aPu}$). Although Endo III processes dHU substrates in two consecutive reactions, DNA glycosylase and AP lyase, only one irreversible step characterized by the rate constant $k_3^\text{aPu}$ was identified. The value of $k_3^\text{aPu}$, 0.054 s$^{-1}$, was 6.7-fold lower than for the AP-aPu substrate, indicating that under our experimental conditions (close to single turnover) the rate is limited by the N-glycosylase reaction, in agreement with the earlier reports (10, 48).

**Conformational Transitions in the Complementary DNA Strand**—To register the conformational transitions in the DNA strand opposite to the lesion, three fluorescent base analogs (Cpy, aPu, and tCO) were tested. In previous work, we have used Cpy and tCO to study conformational dynamics induced in the substrate by DNA glycosylases Fpg (31) and Nei (33), respectively. In the case of Endo III interaction with the dHU-containing duplexes the initial screening experiments have shown that DNA conformational changes were more pronounced if the tricyclic cytosine analog tCO was placed opposite to the damaged base compared with Cpy or aPu reporters (data not shown). Interestingly, earlier reports characterized tCO as a base analog that is highly fluorescent but has a lower sensitivity to the environment in double-stranded DNA in comparison with aPu (49, 50). An aPu residue placed opposite dHU was insensitive to the binding by Endo III. Therefore, tCO located opposite to the lesion was used for the study of conformational transitions in the complementary DNA strand.

**G/tCO Ligand**—Nonspecific binding of the G/tCO ligand by Endo III led to an increase in tCO fluorescence intensity up to 10 s (Fig. 5A). As was suggested for another member of the HhH-GPD structural superfamily, hOGG1 (20), the initial step of DNA binding includes hydrogen bond formation between two helix-invading amino acids, Arg$^{154}$ and Arg$^{204}$ (Arg$^{78}$ and Arg$^{84}$ in *G. stearothermophilus* Endo III, Fig. 3A), and the cyto-
sine base located opposite to the damaged base. Furthermore, as was suggested for Tyr203 and Asn149 of hOGG1 (20), the side chain of Leu81 of Endo III (an equivalent of Tyr203, Fig. 3B) can be partially inserted into the DNA duplex during nonspecific complex formation, and the side chain carbonyl of Gln41 of Endo III (an equivalent of Asn149, Fig. 3B) contacts the base opposite to the lesion site. Therefore, similarly to hOGG1, the tCO fluorescence intensity increase may be due to destabilization and local unwinding of the DNA duplex. Fitting the experimental data to a one-site binding model (Scheme 1), we calculated the values of the forward and reverse rate constants (Table 3). Although a direct comparison between G/tCO and G-aPu nonspecific ligands was precluded by the lack of fluorescent changes in the latter, the rate constant values show that Endo III interacts with G/tCO very slowly, 103-fold slower than with the specific AP-aPu or dHU-aPu substrates (see Table 2).

**F/tCO Ligand**—The interaction of Endo III with F/tCO ligand produced a two-phase change in the fluorescence intensity in the stopped-flow experiments (Fig. 5B). The fast decrease phase was complete by 10 ms, followed by an increase phase up to 5 s. The stopped-flow data were fitted to Scheme 3; the forward and reverse rate constants are presented in Table 3. Interestingly, tCO placed opposite to the lesion site allowed detection of the events occurring earlier during Endo III binding to an F-site than those revealed by aPu reporter placed on the 3'-side of this lesion (compare Figs. 4A and 5B). This observation supports the hypothesis that formation of the initial specific enzyme-DNA recognition complex directly involves both damaged and complementary DNA strand (20). Besides, the rate constants presented in Tables 2 and 3 reveal that when Endo III interacts with the F ligand, the enzyme perturbs the environment of the tCO reporter in the complementary strand 103 times faster in comparison with the environment of the aPu reporter in the F-containing strand, suggesting that Endo III forms first contacts with the complementary DNA strand.

**AP/tCO Substrate**—Upon the interaction with the AP/tCO substrate, the fluorescence of tCO demonstrated a more complex behavior. First it decreased up to 10 ms as in the case of the F/tCO ligand (Fig. 5C). The second phase of decrease in the tCO fluorescence proceeded between 10 ms and 1 s. This two-step drop in the tCO fluorescence presumably reflects productive AP site recognition by Endo III. The β-elimination reaction resulting in the damaged strand cleavage, as well as the product release, proceeded at times >1 s and induced an increase in the
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Opposite Base Specificity of Endo III—It has been reported that the base opposite the lesion may influence the activity of Endo III but the opposite base specificity strongly depends on the lesion: whereas thymine glycol-5,6-dihydrothymine-, formamidopyrimidine-, and N-(2-deoxy-β-D-erythro-pentofuranosyl)-N-3-[2(R)-hydroxyisobutyric acid]-urea-containing substrates show a moderate to pronounced preference for G opposite the lesion, ura, and 5-hydroxycytosine are excised equally well from all pairs (48, 51, 52). We have examined the opposite base specificity of dHU excision by Endo III in a single turnover mode (Fig. 6). The formation of the nicked products was directly detected by PAGE. Endo III was found to moderately discriminate the bases placed opposite to the lesion; the rate and equilibrium constants of the steps estimated from this kinetic scheme are listed in Table 3. In the case of AP substrate the first binding step detected by the tCO fluorescence was ~23-fold faster than the step detected by the aPu fluorescence. Nevertheless, the total association constants K_{bind} are similar for both substrates. The rate constant of the irreversible catalytic step k_{cat}^{tCO} was 0.13 s^{-1}, somewhat lower than k_{cat}^{aPu} = 0.38 s^{-1} obtained for the AP-aPu substrate. Therefore, tCO moderately affects the rate of the catalytic step, presumably due to an increased size of this residue in comparison with the natural cytosine base.

dHU/tCO Substrate—Binding of the dHU/tCO substrate by Endo III induced changes in the tCO fluorescence similar to those observed with the AP/tCO substrate (Fig. 5D). When the base is present, two chemical steps occur after the formation of the catalytically competent complex: cleavage of the N-glycosidic bond and β-elimination reaction. The additional chemical step of N-glycosidic bond cleavage slightly shifts the rising phase of the curves into longer times compared with the AP/tCO substrate. The traces can fit to the minimal kinetic Scheme 2 containing two equilibrium substrate binding steps followed by an irreversible chemical step and then with an equilibrium product release step. The rate constants of the elementary steps estimated from this kinetic scheme are listed in Table 3. It can be seen that k_{3}^{tCO} = 0.077 s^{-1} is very close to the k_{3} value determined by aPu fluorescence (k_{3}^{aPu} = 0.054 s^{-1}). The rate constants corresponding to the binding step are also in a general agreement for both reporters. Therefore, in the case of the dHU substrate the conformational perturbation induced in DNA by Endo III binding occur nearly simultaneously in both strands.

Discussion

Endo III from E. coli is a prototypical member of the endonuclease III structural superfamily of DNA glycosylases, defined according to the presence of two structural elements, a HbH motif and an extended glycine/proline-rich loop containing a catalytic Asp residue (G/P...D loop) (16). Crystal structures have been reported for free Endo III from E. coli (14, 53) and for Endo III from G. stearothermophilus cross-linked to DNA as a reduced Schiff base intermediate or non-covalently bound to F-containing DNA (15). All structures show that Endo III is a two-domain α-helical protein, with one domain organized into a six-helix barrel, and another domain containing an iron-sulfur [4Fe-4S]^{2+} cluster (FeS).

Because the conformational changes in the protein globule of OGG1, another Endo III superfamily member, could be followed by Trp fluorescence with a reasonably good time and amplitude resolution (17, 18, 20), we expected to be able to obtain Trp fluorescence traces for Endo III-DNA interactions as well. E. coli Endo III contains two Trp residues, Trp^{132} and Trp^{179}. The latter is buried inside the FeS domain, tightly packed against the α-helix, and is unlikely to be considerably mobile in the course of DNA substrate recognition and processing. Trp^{132}, however, is located in an inter-domain loop on the surface of the protein opposing the DNA-binding groove, and we anticipated that DNA binding would induce significant conformational changes to be observed by Trp fluorescence. Our failure to detect Trp fluorescence changes indicates that either Endo III binds DNA with minimal domain movement, or the solvent exposure of Trp^{132} changes little upon DNA binding. Our preliminary structure of E. coli Endo III cross-link to DNA shows no great deviations from the structure of the wild-type protein (Co root mean square deviation: 2.12-Å overall, 2.09-Å six-helix barrel domain, 1.27-Å FeS domain) and shifted but still widely exposed side chain of Trp^{132}, so both factors probably contribute to the observed lack of Trp fluorescence change.

DNA in complex with both G. stearothermophilus Endo III (15) and E. coli Endo III^{4} is grossly distorted at the site of the

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4 D. O. Zharkov, unpublished data.
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Eco-Endo III: -MKAKGRLLEILTRLRENHPPHTELNFSSPPFELLIVLLQAGIVYOKTAKLKYPVAN 59
Bst-Endo III: MLTKQIRQYCLDEMAKMPDHELARCEVLHRNPFFELLIAVL5QCTDAVYKVUTKRLFEKYR 60

Eco-Endo III: TPAAMELGEVGGKVTITLGLNKAENIKRCIRQLEQNVEGVEDRAMEAFDCF 119
Bst-Endo III: TPHDYAVPPLREIQDsignIGSKKHQKNNQKLCANDLQKQVGEVPRDDKLQ 120

Eco-Endo III: CTANVVLNTAFGWPIAVYH1T1V1VNCMTQFAPKG-KNVQVEEKLKLVRNAEMWV 178
Bst-Endo III: CTANVVLVSAGFPAIVDFWVVRV1SVNLQCFCWDDVVLEEPYVMMK1PKEWISHR 180

Bst-Endo III: LIILHGGRTICARKPRGCNCICDLEIYVEKKYDI1 211
Bst-Endo III: MFFGFRYRKAQSPQPSFPLLHLRGRKRRKREWKEAAQK 223

FIGURE 7. Alignment of sequences of Endo III from E. coli (Eco-Endo III) and G. stearothermophilus (Bst-Endo III). Motifs responsible for DNA distortion are boxed green. The catalytic Lys and Asp residues are underlined. Cys residues forming the FeS cluster are labeled cyan. Helices αA-αP are shown based on the Bst-Endo III structure (Protein Data Bank code 1ORN, (15)) and are alternatively marked in black and red to show the borders. Asterisks indicate identical residues, colons, conserved residues; dots, residues with at least some physicochemical properties conserved. The alignment was produced with Clustal Omega (65).

lesion, with the DNA axis kinked ~55°, the minor groove widely open, and the damaged nucleotide everted from the helix into the active site pocket of the enzyme. Four distinct structural elements of the protein are responsible for this distortion, discussed below for the published G. stearothermophilus structure (refer to the alignment in Fig. 7 for the corresponding boxed green and red residues, colons, conserved residues; dots, residues with at least some physicochemical properties conserved. The alignment was produced with Clustal Omega (65).

This arrangement of DNA and protein residues provides insights into the conformational transition dynamics associated with the changes in fluorescence of the reporters incorporated into DNA (Fig. 8). Fluorescence of synthetic base analog aPu is quenched in a less polar environment, so the decrease in the fluorescence of this reporter at the initial phases of reaction with the F ligand and AP and dHU substrates is obvious associated with the insertion of Gln41 into the space vacated by the everted nucleotide and stacking of its amide group against aPu. Multiple solution NMR studies of DNA duplexes show that F and AP tend to be intrahelical or slightly displaced toward the major or minor groove when the opposite base is well stacked (reviewed in Ref. 54). In our case, when the opposite guanine base is in the ...GGC... context, the abasic nucleotides are likely intrahelical and therefore the adjacent bases are solvent accessible in free DNA, so Endo III binding and Gln41 stacking would decrease the fluorescence from the baseline. No structure of dHU-containing DNA has been reported but, as other 5,6-saturated pyrimidine lesions (54), it is probably displaced or partially extrahelical, also exposing the adjacent bases to the solvent in free DNA. Fast increase in the aPu fluorescence in the case of the dHU substrate indicates fixation of the damaged base in the extrahelical state. However, longer times of fluorescence decrease with dHU indicate that the insertion of Gln41 in the DNA void after eversion of this lesion in the active site is energetically less favorable compared with AP substrate.

When another fluorescent reporter, tcC5, was incorporated opposite to the lesion, a single decrease in its fluorescence for the F ligand (Fig. 5A) and a two-phase decrease with the AP and dHU substrates (Fig. 5, B and C) were recorded. On the contrary, binding of the G ligand as well as the second step of F ligand binding lead to an increase in tcC5 fluorescence. The αF-αG motif of Endo III binds the undamaged strand exclusively through the backbone carbonyls and is flexible enough to accommodate G or A in two different conformations (15), so tcC5 will be presumably bound without significantly disrupting functionally important interactions with the undamaged strand. In the same vein, although Endo III showed some specificity for a base opposite the lesion, it was capable of efficiently exciting dHU paired with all nucleobases (Fig. 6), unlike the Endo III superfamily member hOGG1, which strongly discriminates against adenine opposite the lesion and forms several specific contacts with the orphaned base (22).

The variations in the quantum yield of tcC5 are not straightforward to interpret; it seems to be stacking-dependent in single-stranded DNA but relatively invariable in double-stranded DNA, and, if tcC5 is located between two G bases, the fluorescence in single-stranded DNA is quenched relative to double-stranded DNA (50). Therefore, we hypothesize that the first decrease in fluorescence in cases of F ligand and AP and dHU substrates may be associated with DNA kinking upon Endo III
binding, or with Leu81 wedging and eversion of the damaged nucleotide from DNA (Fig. 8). Notably, these events seem to be also displayed in the aPu fluorescence traces (Fig. 4, A–C) before the insertion of Gin41, indicating that the initial stages of DNA distortion do not require active helix penetration by the αβ–αC turn. The shapes of tCO fluorescence traces (Fig. 5, A–D) suggest that the increase in tCO fluorescence upon non-specific binding may also be associated with partial DNA melting. As can be seen from Tables 2 and 3, for F- and AP-DNA binding \( k_{1\text{aPu}} < k_{1\text{tCo}} \), indicating that this disruption of DNA base pairing proceeds after the formation of initial contacts with Endo III. Such disturbance of DNA conformation in both specific and non-specific complexes may result from the attempts of the enzyme to flip out the sampled base independently of whether it is damaged or not. Active eversion of undamaged bases from DNA in the process of lesion search was also shown for hOGG1 (23, 24). Also, in all available structures of DNA glycosylases with undamaged DNA (23, 55–60) the wedging residue of the enzyme is already inserted in DNA, so Leu81 of Endo III might be expected to behave in the same way as it was recently shown by single-molecules studies in Refs. 61 and 62. Therefore, the other possible molecular event causing a tCO fluorescence increase in the first phase of non-specific DNA binding might be insertion of Leu81 as a part of lesion search, affecting the environment of tCO in fully stacked undamaged DNA in another way than in the case of already destabilized damaged DNA.

The second phase of F ligand binding induced an increase in the tCO fluorescence signal (Fig. 5, C and D) led to a decrease in the tCO fluorescence signal. Therefore, this stage is not likely to arise from interactions with the αF–αG motif, because they are identical in the Endo III complex with the F ligand and with the reduced Schiff base intermediate (15). Rather, we suggest that the extended size of the tCO base allows it to partially overlap with the bases in the damaged strand and respond to the structural changes occurring when DNA around the lesion adopts a catalytically competent conformation. A comparison of DNA structure in close vicinity of the lesions in the cases of F and reduced Schiff base (15) indicates that F is pulled deeper into the active site pocket of the enzyme, and the space between the adjacent bases is narrower with F. The relaxation of this DNA distortion upon pre-catalytic conformational adjustment may ease stacking with the protruding part of tCO thus causing a further decrease in its fluorescence.

A comparison of kinetic data obtained by aPu and tCO fluorescence measurements (compare \( k_{3\text{aPu}} \) and \( k_{3\text{tCo}} \) values, Table 2 and 3) together with the lack of additional product accumulation after an alkaline treatment support the conclusion that the rate-limiting step of Endo III catalysis is base excision rather than substrate binding or DNA backbone cleavage. This may be related with a need to adjust the active site conformation to a wide spectrum of oxidatively damaged bases recognized and removed by Endo III (9, 11, 26, 52), precluding the existence of a stiff preformed active site highly specific for one particular substrate.

**Conclusion**

The kinetic analysis of conformational transitions in DNA ligands and substrates for *E. coli* Endo III DNA glycosylase,
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based on stopped-flow fluorescence traces of reporter base analogs aPu and tC\textsuperscript{O}, located in the damaged strand and in the opposite chain, respectively, reveals the dynamics of different regions of DNA around the damaged site during the interaction with Endo III and opens a window into the mechanism of enzyme catalysis (Fig. 8). As in the case of previously studied DNA glycosylases, E. coli Fpg (28, 29, 31, 63) and human OGG1 (20, 21), binding of Endo III to DNA begins most likely with an insertion of the wedge amino acids, Gln\textsuperscript{41} and Leu\textsuperscript{81}, into the helix (61, 62). Insertion of other amino acids and eversion of the damaged nucleotide into the active site proceed at the next stage of specific lesion recognition, resulting in “stapling” of the enzyme on DNA. After that the active site is adjusted to the catalytically competent conformation, and the catalytic steps end up in the cleavage of the N-glycosidic bond and β-elimination.

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