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Photochemical cross-linking of *Escherichia coli* Fpg protein to DNA duplexes containing phenyl(trifluoromethyl)diazirine groups

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Formamidopyrimidine-DNA glycosylase (Fpg protein) of *Escherichia coli* is a DNA repair enzyme that excises oxidized purine bases, most notably the mutagenic 7-hydro-8-oxoguanine, from damaged DNA. In order to identify specific contacts between nucleobases of DNA and amino acids from the *E. coli* Fpg protein, photochemical cross-linking was employed using new reactive DNA duplexes containing 5-[4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl]-2′-deoxyuridine dU* residues near the 7-hydro-8-oxoguanosine (oxoG) lesion. The Fpg protein was found to bind specifically and tightly to the modified DNA duplexes and to incise them. The nicking efficiency of the DNA duplex containing a dU* residue 5′ to the oxoG was higher as compared to oxidized native DNA. The conditions for the photochemical cross-linking of the reactive DNA duplexes and the Fpg protein have been optimized to yield as high as 10% of the cross-linked product. Our results suggest that the Fpg protein forms contacts with two nucleosides, one 5′ adjacent to oxoG and the other 5′ adjacent to the cytidine residue pairing with oxoG in the other strand. The approaches developed may be applicable to pro- and eukaryotic homologues of the *E. coli* Fpg protein as well as to other repair enzymes.

Keywords: formamidopyrimidine-DNA glycosylase; modified DNA duplexes; 7-hydro-8-oxoguanosine; 5-[4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl]-2′-deoxyuridine; photochemical cross-linking.

Derivatives of nucleic acids containing photolabile carbene-generating aryl(trifluoromethyl)diazirine groups are conveniently used to identify specific nucleic acid·nucleic acid and nucleic acid·protein interactions [1–5]. These derivatives have a number of essential merits. First, they produce highly reactive carbene, which breaks even aliphatic C–H bonds. Second, the lifetime of carbene is on a nanosecond timescale. Third, photolysis proceeds at a relatively high light wavelength (350–360 nm) that does not cause damage to biological molecules. Finally, these derivatives may be handled under moderate laboratory illumination. These reagents have been successfully employed to investigate RNA·RNA and RNA·protein interactions in ribosomes [1], and to ascertain specific contacts between DNA and some DNA-recognizing proteins, such as the restriction-modification enzymes *Eco*RII and *Mva*I [2], recombinant rat DNA polymerase β [3], the large subunit of human immunodeficiency virus reverse transcriptase [4], yeast RNA polymerase and others [5].

Escherichia coli formamidopyrimidine-DNA glycosylase (Fpg protein) is a DNA repair enzyme that catalyzes the removal of oxidized purine bases from damaged DNA and cleaves the DNA strand [6]. 7-Hydro-8-oxoguanine is the major mutagenic base produced in DNA by reactive oxygen species that are generated by cellular metabolism, cell injury and exposure to physical and chemical oxygen radical-forming agents [7]. It is a miscoding lesion because it pairs preferentially with adenine rather than cytosine and induces GC → TA transversions *in vivo* and *in vitro* [8]. The physiological function of the Fpg protein is to prevent the mutagenic action of oxoG residues in DNA and to maintain genetic integrity. Three-dimensional structures of the complexes formed by *Lactococcus lactis*, *Bacillus stearothermophilus* and *E. coli* Fpg proteins with abasic DNA duplexes have recently been obtained using X-ray crystallography [9–11]. However, despite this success, further biochemical data are still needed to understand the dynamics of the interaction of the Fpg protein active-site residues with various substrates. Valuable information can be obtained by using a variety of cross-linking techniques applicable to nucleic acid·protein systems. Previously, we used chemical cross-linking to identify specific contacts between *E. coli* Fpg protein amino acid residues and DNA phosphate groups [12]. Here, we use photochemical cross-linking to ascertain specific contacts between the Fpg protein and the nucleosides adjacent to oxoG. To achieve this, modified

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Abbreviations: EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; Fpg protein, formamidopyrimidine-DNA glycosylase; $K_D$app, apparent dissociation constant for the binding of the Fpg protein to the modified duplexes; oxoG, 7-hydro-8-oxoguanosine; TFMDPh, 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl; dU*, 5-[4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl]-2′-deoxyuridine.

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DNA duplexes containing 5-[4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl]-2′-deoxyuridine (dU*) residues 5′ to the oxoG lesion or 5′ to the cytidine residue of the other strand, forming a base pair with oxoG, were prepared. To our knowledge, this is the first time that double-stranded oligonucleotides containing reactive 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl (TFMDPh) groups have been used to study interactions with DNA repair enzymes.

Materials and methods

Oligonucleotides

Oligonucleotides (1)–(6) and DNA duplexes I–IV, used in this study, are depicted in Fig. 1. Oligonucleotides (1)–(4), forming DNA duplexes II–IV, were synthesized using a standard phosphoramidite procedure in an Applied Biosystems 380 B DNA synthesizer, as described by Matteucci *et al.* [13]. Modified oligonucleotides (2) and (6), containing oxoG, were prepared using commercial 3′-phosphoramidite of modified 2′-deoxyguanosine. Synthesis of modified oligonucleotides (3) and (5), containing dU*, was performed as described by Topin *et al.* [2]. Oligonucleotide (5), with a 3′-terminal phosphate group, was obtained according to Purmal *et al.* [14]. The oligonucleotides were 5′ end-labelled with T4 polynucleotide kinase and [γ-^{32}P]dATP following the standard procedure [15]. The concentrations of oligonucleotides were determined spectrophotometrically.

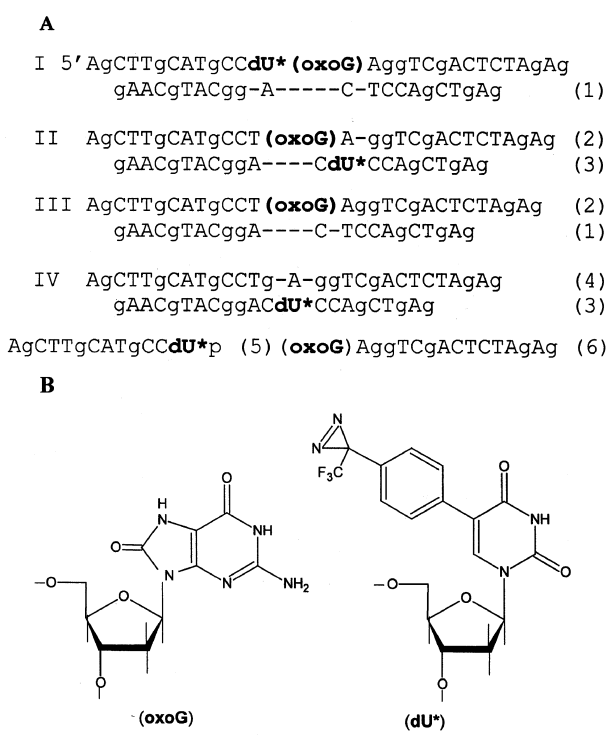

Fig. 1. Structures of (A) oligonucleotides and modified DNA duplexes and (B) modified nucleosides used in this study. Figures in Roman indicate the numbers of corresponding DNA duplexes; figures in Arabic indicate the numbers of corresponding oligonucleotides.

Chemical ligation of oligonucleotides

An equimolar mixture of oligonucleotides (1), (5) and (6), forming nicked DNA duplex I (the total nucleotide concentration was 10 mM), was incubated at 75 °C for 2 min in 0.05 M Mes/NaOH buffer, pH 6.0, containing 0.02 M $MgCl_2$, and slowly cooled for 2 h. Then, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) was added to a concentration of 0.2 M. The reaction was carried out at 20 °C for 72 h in the dark. The ligation product was isolated by PAGE (20% denaturing gel), followed by elution with 2 M $LiClO_4$, precipitation with five volumes of acetone and reprecipitation from 2 M $LiClO_4$ by a further two precipitations with 10 volumes of acetone.

Gel retardation assay

Binding reactions were performed at 0 °C for 5 min. The incubation mixture (20 μL final volume) contained 25 mM Hepes/KOH, pH 7.6, 100 mM KCl, 5 mM β-mercaptoethanol, 2 mM Na_2EDTA, 0.1% (w/v) BSA, 6% (v/v) glycerol, 50–70 pM [^{32}P]-labelled DNA duplex expressed as the oxoG concentration and 0.5–10 nM Fpg protein. Samples were subjected to nondenaturing PAGE (10% gel) and were visualized by autoradiography. The radioactivity of gel slices was determined by Cerenkov counting. The yield of the complex was calculated as the ratio of shifted band radioactivity to the total radioactivity of the loaded sample. The apparent dissociation constants were determined as described by Boiteux *et al.* [16].

Assays for enzymatic activity

The standard assay (12 μL final volume) contained 25 mM Hepes/KOH, pH 7.6, 100 mM KCl, 5 mM β-mercaptoethanol, 2 mM Na_2EDTA, 0.1% (w/v) BSA, 6% (v/v) glycerol, 0.7 nM [^{32}P]-labelled oxoG-containing duplexes, expressed as the oxoG residues and 5 nM enzyme. The incubation was performed at 37 °C. The reaction was stopped by the addition of 3 μL of formamide dye to 2 μL of solution. The mixture was heated at 90 °C for 3 min and loaded onto a denaturing 20% polyacrylamide gel containing 7 M urea.

Photochemical cross-linking experiments

The Fpg protein (6 nM) and [^{32}P]-labelled DNA duplexes I or II (concentration of 5–10 nM per duplex) were incubated in 20 μL of the binding buffer at 0 °C for 5 min. To analyse the photochemical cross-linking reaction, the samples were placed in microwell plates (Fisher Life Science) and irradiated with ultraviolet (UV) light (366 nm wavelength) for 30 min on ice using a high-intensity UV lamp (model UVGL-58). The reaction progress was followed by 0.1% SDS/12% PAGE [17] after heating the samples in 0.1% SDS/2-mercaptoethanol solution at 95 °C. The gels were analyzed by autoradiography and silver staining. Equal mobilities of the radioactive and the protein-containing bands indicated covalent attachment of DNA to the enzyme. The yield of the photochemical cross-linking reaction was calculated as the ratio of the covalent conjugate radioactivity to the total radioactivity of the conjugate and unbound DNA.

Results and discussion

Design of modified DNA duplexes

E. coli Fpg protein recognizes a hexanucleotide sequence with oxoG in the middle in the lesion-bearing DNA strand and specifically binds to it and the oxoG-pairing residue of the other strand [18]. This residue is thought to be everted from the double helix during catalysis [19]. We propose that neighbouring nucleosides are also involved in the formation of the enzyme–substrate complex. In order to identify specific contacts between the Fpg protein and the nucleosides located near the oxoG lesion in DNA, modified DNA duplexes containing dU* residues near oxoG were prepared (Fig. 1). The dU* residue, bearing a photolabile TFMDPh group, was introduced into the oxoG-containing strand of a 29/22-mer DNA duplex 5′ to oxoG (duplex I) or 5′ to the oxoG-pairing cytidine residue of the other strand (duplex II). DNA duplex III did not contain any dU* residue and was used to estimate the effect of the TFMDPh group on DNA duplex binding to the Fpg protein. DNA duplex IV was similar to duplex II, but contained a guanosine residue instead of oxoG. This duplex was used to check whether the binding of DNA duplexes I and II to the Fpg protein was specific.

A 29-mer oligonucleotide used to prepare DNA duplex I, and containing both the oxoG and the dU* residues, was obtained by a template-induced chemical ligation of oligonucleotide (5), carrying a 3′-end phosphate group, to oligonucleotide (6), bearing a 5′-end OH group, as described in the Materials and methods. The ligation efficiency was as high as 50%.

DNA duplexes I–IV were formed after annealing of the corresponding 29-mer oligonucleotides with an equimolar amount of a 22-mer complementary oligonucleotide.

Binding of the modified DNA duplexes to the Fpg protein

DNA duplexes I–III were tested for binding to the Fpg protein in order to determine whether it can specifically recognize dU*-bearing modified DNA duplexes. The binding was detected by gel-retardation shift assay. We found that the Fpg protein recognizes and specifically binds all the tested duplexes with high efficiency. Figure 2 illustrates a single retardation band, which indicates complex formation between DNA duplex II and the Fpg protein. The intensity of the retardation band increased with increasing Fpg protein concentration. The binding reaction was performed at a low temperature (0 °C) because no significant cleavage was observed in these conditions.

The apparent dissociation constant, $K_D$app, for the binding of the Fpg protein to the modified duplexes, was estimated from the gel-retardation data, as described by Boiteux *et al.* [16]. The $K_D$app values obtained were 1.0 ± 0.2, 1.2 ± 0.3 and 2.0 ± 0.3-nM for DNA duplexes I, II and III, respectively. Thus, the binding efficiency of the reactive DNA duplexes I and II was similar to the binding efficiency of DNA duplex III, which has the same sequence but contains no photolabile TFMDPh group. The results obtained indicate that introduction of the TFMDPh group in close proximity to the oxoG residue has no effect on the recognition and binding of DNA duplexes by the Fpg protein.

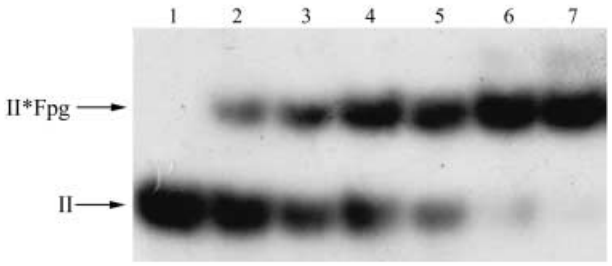

Fig. 2. Binding of DNA duplex II to the formamidopyrimidine-DNA glycosylase (Fpg protein). Autoradiogram from a gel retardation assay using 50 pM of [^{32}P]-labelled DNA duplex II containing a 5-[4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl]-2′-deoxyuridine (dU*) residue in the absence (lane 1) or presence of 0.5, 1.0, 2.0, 4.0, 6.0, or 8.0 nM of the Fpg protein (lanes 2–7, respectively). The structure of duplex II is depicted in Fig. 1; for experimental conditions see the Materials and methods.

Specificity of Fpg protein binding

The interaction between the Fpg protein and modified DNA duplexes I and II was shown to be specific by two independent criteria. First, a 150-fold excess of unlabelled DNA duplex II almost completely suppressed the binding of the labelled DNA duplex II (Fig. 3). By contrast, duplex IV, formed by oligonucleotides (3) and (4) having identical nucleotide sequences but containing no oxoG, did not

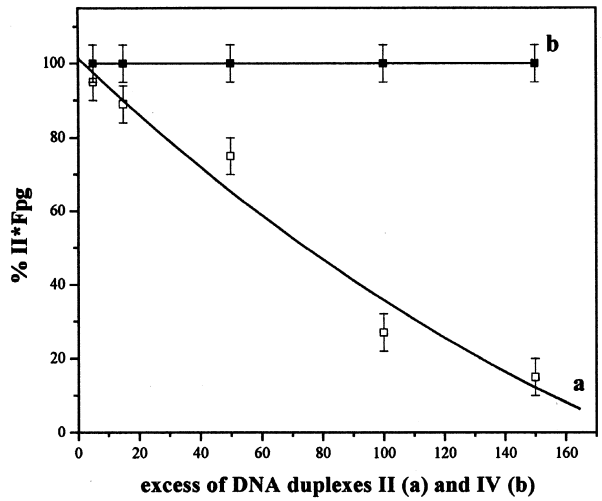

Fig. 3. Suppression of [^{32}P]-labelled DNA duplex II binding to the formamidopyrimidine-DNA glycosylase (Fpg protein) by an excess of unlabelled DNA duplexes II (a) and IV (b). The binding assay was carried out with 8.0 nM Fpg protein and 50 pM [^{32}P]-labelled DNA duplex II containing a 5-[4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl]-2′-deoxyuridine (dU*) residue in the presence of 5-, 15-, 50-, 100- and 150-fold excess of unlabelled DNA duplexes II and IV. The experiment was repeated three times and gave reproducible results.

compete with the labelled modified duplex II for the Fpg protein under the same conditions (Fig. 3).

Assays for enzymatic activity

The Fpg protein is known to release oxoG residues from DNA and cleaves 3′- and 5′-phosphodiester bonds via successive β- and δ-elimination reactions [20]. To investigate the influence of the TFMDPh group on the substrate properties of modified DNA duplexes I and II, their catalytic incision by the Fpg protein was tested. Figure 4 shows time-course data of the cleavage reaction with DNA duplexes I–III. DNA duplexes I and II are incised by the enzyme. The efficiency of DNA incision was dependent on the position of the TFMDPh group and was higher for DNA duplex I, which in this group is 5′ adjacent to the oxoG residue. This may result from conformational changes induced by the TFMDPh group in the substrate structure.

Photochemical cross-linking experiments

In order to ascertain specific contacts between the Fpg protein and the nucleosides located in the vicinity of the oxoG residue, a photochemical cross-linking procedure was employed. Specific complexes between the Fpg protein and radiolabelled DNA duplexes I and II were formed, as described in the Materials and methods. UV-irradiation (366 nm wavelength) of both complexes resulted in DNA–protein cross-linking (Fig. 5). The molecular masses of the complexes formed by DNA duplexes I and II were estimated from the mobilities of the retarded species in 0.1% SDS/12% PAGE as ≈ 41 and ≈ 38 kDa, respectively, corresponding to 30.2 kDa protein linked to 9.6 kDa 29-mer and 7.5 kDa 22-mer dU*-containing oligonucleotides. Photochemical cross-linking appeared to be specific because Fpg protein binding to DNA duplexes I and II resulted in only one specific DNA·Fpg protein complex (see above). Cross-linking efficiency was as high, being 10% for DNA duplex I containing the photolabile group 5′ to oxoG, and 2% for DNA duplex II. This difference may be explained by variations in the nature and, consequently, the accessibility of the amino acid residues participating in the complex formation with DNA to the reactive TFMDPh.

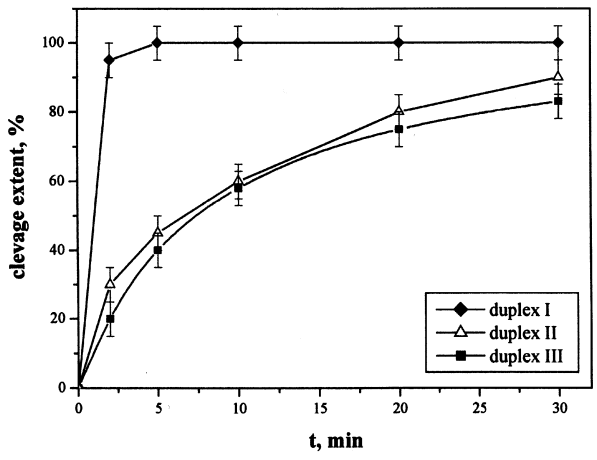

Fig. 4. Time-course of cleavage of modified DNA duplexes I–III by the formamidopyrimidine-DNA glycosylase (Fpg protein). The extent of cleavage was determined by PAGE as the ratio of the incised DNA radioactivity to the total radioactivity of incised and native DNA. The results of three independent experiments agreed within 5%.

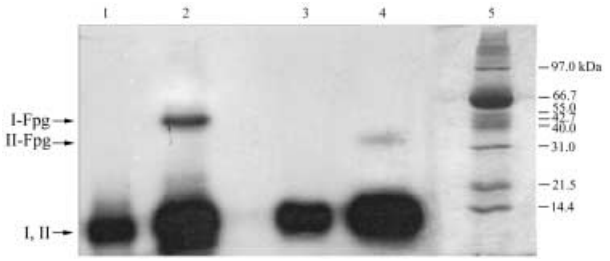

Fig. 5. SDS/PAGE analysis of the photochemical cross-linking reactions. The reaction was performed, as described in Materials and methods, for 30 min at 0 °C. An autoradiogram of a 12% SDS gel showing the cross-linking of DNA duplexes I and II to the formamidopyrimidine-DNA glycosylase (Fpg protein) (lanes 2 and 4, respectively) is presented. Lanes 1 and 3 show DNA duplexes I and II, respectively, in the absence of the Fpg protein. Molecular masses of the standard proteins (lane 5) are indicated on the right.

Based on the X-ray crystallographic data on Fpg protein·abasic DNA complexes [10,11], the most likely candidate for the Fpg protein residue contacting the nucleoside 5′ adjacent to oxoG is a highly conserved Arg258 from a zinc-finger motif. This residue is lodged between two successive phosphates of DNA, which are located on the 5′ and 3′ sides of the lesion, and is specifically bound to both. Tyr236, which interacts with the phosphate group 5′ adjacent to oxoG, may be also involved, insofar as carbene generated by the TFMDPh group is more readily inserted into O–H bonds in comparison with C–H bonds [21]. Other possible candidates include Met73, Arg108 and Phe110, which may enter the DNA helix to occupy the space freed upon oxoG eversion [10,11]. As follows from the X-ray data, Met73 makes van der Waals contacts with the lesion sugar atoms, while Arg108 forms a number of hydrogen bonds to the Watson–Crick face of the estranged cytidine and a π-stacking over the nucleotide base 5′ adjacent to the lesion [11]. We further suggest that Phe110 is involved in a stacking interaction with the aromatic ring of the TFMDPh group. The complementary DNA strand forms few interactions with the enzyme, and the amino acid residues involved are not conserved [11]. The nucleoside 5′ adjacent to the oxoG-pairing cytosine residue is likely to interact with Phe110 or Arg109. Phe110 invades the DNA helix on the 5′ side of the estranged cytosine, simultaneously making an edge–face interaction with the estranged cytosine and a face-to-face π interaction with the pyrimidine ring of the 5′ neighbouring nucleotide [10,11].

In summary, we ascertain specific contacts of two nucleosides, 5′ adjacent to oxoG and 5′ adjacent to the oxoG-paired cytidine residue, with amino acid residues of *E. coli* Fpg protein. These results, together with data from ongoing studies of the Fpg protein and its pro- and eukaryote homologues, will help to further elucidate the molecular mechanism of DNA repair. The approaches developed can be employed in the studies of other DNA repair enzymes.

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