# Recognition and Kinetics for Excision of a Base Lesion within Clustered DNA Damage by the *Escherichia coli* Proteins Fpg and Nth

Marie-Hélène David-Cordonnier,[‡] Jacques Laval,[§] and Peter O'Neill*,[‡]

*Medical Research Council, Radiation and Genome Stability Unit, Harwell, Didcot, Oxon OX11 0RD, United Kingdom, and UMR 8335 CNRS, Groupe Réparation de l'ADN, Institut Gustave Roussy, 94805 Villejuif Cedex, France*

*Received November 13, 2000; Revised Manuscript Received March 5, 2001*

ABSTRACT: Ionizing radiation and radiomimetic anticancer agents induce clustered DNA damages that are thought to lead to deleterious biological consequences, due to the challenge that clustered damage may present to the repair machinery of the cell. Specific oligonucleotides, containing either dihydrothymine (DHT) or 7,8-dihydro-8-oxoguanine (8-oxoG) opposite to specific lesions at defined positions on the complementary strand, have been used to determine the kinetic constants, $K_M$, $k_{cat}$, and specificity constants, for excision of DHT and 8-oxoG by the *Escherichia coli* base excision repair proteins, endonuclease III (Nth) and formamidopyrimidine glycosylase (Fpg), respectively. For excision of DHT opposite to 8-oxoadenine by Nth or Fpg proteins, or 8-oxoG opposite to 8-oxoG by Fpg, the major change in the specificity constant occurs when the second lesion on the complementary strand is one base to the site opposite to DHT or 8-oxoG. The specificity constants for excision of DHT or 8-oxoG by both proteins are reduced by up to 2 orders of magnitude when an abasic site or a strand break is opposite on the complementary strand. Whereas the values of $K_M$ are only slightly affected by the presence of a second lesion, the major change is seen as a reduction in the values of $k_{cat}$. The binding of Fpg protein to oligonucleotides containing 8-oxoG is inhibited, particularly when a single strand break is near to 8-oxoG on the complementary strand. It is inferred that not only the binding affinity of Fpg protein to the base lesion but also the rate of excision of the damaged base is reduced by the presence of another lesion, particularly a single strand break or an AP site on the complementary strand.

Radiation and radiomimetic anticancer drugs, such as bleomycin, induce high levels of clustered DNA damage, in which two or more elementary lesions are induced within one or two helical turns of the DNA (*1*, *2*). Clustered DNA damage may comprise multiple damaged bases or a base lesion opposite to a strand break. The complexity of radiation-induced clustered DNA damage increases on increasing the ionizing density of the radiation (LET)[1] (*3*, *4*), and the increased complexity may compromise the repair machinery of the cell. Therefore, clustered DNA damage is thought to be responsible for the deleterious biological consequences of radiation. For instance, clustered lesions in DNA can potentially remain unrepaired or be converted into a double strand break which, if unrepaired, may lead to cell death. The poor efficiency of repair of a clustered DNA damage compared with that for a single lesion may lead to a mutation. Furthermore, clustered damages have been detected in cells at very low doses of radiation (*5*).

DNA glycosylases, such as bacterial Nth protein (also called endonuclease III) and formamidopyrimidine DNA glycosylase (Fpg), are involved in the initial step of base excision repair (BER) to remove specific base substrates from DNA (*6*−*8*), leaving an abasic (AP) site. The AP site is subsequently cleaved by the AP lyase activity of these proteins, giving a single strand break (SSB) in DNA. Nth protein excises mainly ring-saturated pyrimidines (e.g., DHT and thymine glycol) by a $\beta$-elimination process (*9*−*12*), whereas Fpg protein excises not only 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (Fapy) (*13*) and 7,8-dihydro-8-oxo-2′-deoxyguanine (8-oxoG) (*14*, *15*) but also DHT (*16*) by a $\beta$−$\delta$-elimination process.

A few studies have shown that the efficiency of conversion of a single lesion into a SSB by prokaryotic BER proteins, such as Nth and Fpg proteins, is significantly reduced when the lesion is present within a clustered DNA damage (*16*−*19*). For instance, the overall efficiency of excision of DHT, a DNA radiolytic product induced under reductive conditions, by *Escherichia coli* Nth protein is significantly reduced in the presence of an AP site at particular positions on the complementary strand to DHT (*16*, *17*). In contrast, the presence of a base lesion such as 8-oxoA or 8-oxoG, the latter lesion induced in DNA radiolytically and through oxidative metabolism, has little effect, even when positioned one base 5′ to the site opposite to DHT. The presence of DHT or uracil at any of the tested positions on the complementary strand to DHT has no effect on the efficiency of excision of DHT by Nth (*16*, *19*). The efficiency of excision of DHT by the bacterial Fpg protein is greatly

* To whom correspondence should be addressed. E-mail: p.oneill@har.mrc.ac.uk. Tel: +44 1235834393. Fax: +44 1235834776.

[‡] Medical Research Council, Radiation and Genome Stability Unit.

[§] UMR 8335 CNRS, Groupe Réparation de l'ADN, Institut Gustave Roussy.

[1] Abbreviations: DHT, dihydrothymine; AP, apurinic/apyrimidic site; 8-oxoA, 7,8-dihydro-8-oxoadenine; 8-oxoG, 7,8-dihydro-8-oxoguanine; LET, linear energy transfer; bp, base pair; SSB, single strand break; BER, base excision repair.

10.1021/bi002605d CCC: $20.00 
Published on Web 04/17/2001

affected by the presence of an AP site on the complementary strand (up to 50-fold inhibition; *16*) whereas the presence of 8-oxoA slightly enhances the efficiency of excision of DHT by Fpg protein but only when positioned one base 3′ to the site opposite to DHT.

Whether this reduced efficiency of removal of a damaged lesion within a clustered DNA damage is due to reduced recognition and/or catalytic activity of the enzyme is as yet not known. The purpose of this study was to determine the catalytic constants of Nth and Fpg proteins for processing DHT and of Fpg for processing 8-oxoG, as substrates within clustered damage. To date, the catalytic constants for excision of 8-oxoG opposite to cytosine by the Fpg protein have been determined (*20*−*23*) but not in the presence of a neighboring lesion on the complementary strand. From the kinetics of removal of base lesions present within various types of clustered DNA damage and the corresponding Fpg/8-oxoG binding efficiencies, we have assessed whether the inhibition in SSB formation, previously reported (*16*), is due to inhibition of binding and/or subsequent excision of the base lesion by the proteins.

## EXPERIMENTAL PROCEDURES

*Substrate Oligonucleotides*. The 40-mer oligonucleotides were purchased from Genosys. To obtain double-stranded oligonucleotides containing 8-oxoA opposite to DHT, four different sequences of strand 1 were used containing DHT at defined positions from −3 to +3. Position −3: 5′-CTCTTAGTCAGGAATA(DHT)GTTTCTATGCTGGGAGCAAAGGC-3′. Position −1: 5′-CTCTTAGTCAGGAATATG(DHT)TTCTATGCTGGGAGCAAAGGC-3′. Position +1: 5′-CTCTTAGTCAGGAATATGTT(DHT)CTATGCTGGGAGCAAAGGC-3′. Position +3: 5′-CTCTTAGTCAGGAATATGTTTC(DHT)ATGCTGGGAGCAAAGGC-3′. The DHT-containing oligonucleotides were 5′-end-labeled with $^{32}$P by incubation with 10 units of T4 polynucleotide kinase (GIBCO BRL) and 50 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol, 10 mCi/mL, NEN Dupont) for 1 h in 25 $\mu$L of the recommended buffer at 37 °C. Following purification of these single-stranded oligonucleotides on a 12% denaturing polyacrylamide gel, run for 1 h at 80 W, the labeled oligonucleotide was hybridized with 1.5-fold excess of the purified, non-radio-labeled complementary strand containing 8-oxoA at the following site, e.g., 5′-GCCTTTGCTCCCAGCATAGA(8-oxoA)ACATATTCCTGACTAAGAG-3′, or to the control oligonucleotide, which does not contain a damaged site, 5′-GCCTTTGCTCCCAGCATAGAAACATATTCCTGACTAAGAG-3′. The solutions for the hybridization reaction were heated to 80 °C for a few minutes and then allowed to cool slowly to room temperature over ∼2 h. The double-stranded oligonucleotides were then purified in a native 10% polyacrylamide gel, run for 3 h at 300 V in TBE (1×). All the oligonucleotides subsequently prepared have been gel purified as described above, unless otherwise stated.

For preparation of oligonucleotides containing DHT opposite to an AP site at defined positions on the complementary strand, strand 1 containing DHT at a fixed site in the following sequence, 5′-GCCTTTGCTCCCAGCATAGA-(DHT)ACATATTCCTGACTAAGAG-3′, was 5′-end-labeled with $^{32}$P as described above and hybridized with strand 2 containing either thymine, as control, or uracil at defined positions opposite to DHT: control, 5′-CTCTTAGTCAGGAATATGTATCTATGCTGGGAGCAAAGGC-3′; position −5, 5′-CTCTTAGTCAGGAAUATGTATCTATGCTGGGAGCAAAGGC-3′; position −3, 5′-CTCTTAGTCAGGAATAUGTATCTATGCTGGGAGCAAAGGC-3′; position −1, 5′-CTCTTAGTCAGGAATATGUATCTATGCTGGGAGCAAAGGC-3′; position +1, 5′-CTCTTAGTCAGGAATATGTAUCTATGCTGGGAGCAAAGGC-3′; position +3, 5′-CTCTTAGTCAGGAATATGTATCUATGCTGGGAGCAAAGGC-3′; position +5, 5′-CTCTTAGTCAGGAATATGTATCTAUGCTGGGAGCAAAGGC-3′; position +8, 5′-CTCTTAGTCAGGAATATGTATCTATGCUGGGAGCAAAGGC-3′. The gel-purified, double-stranded oligonucleotides were treated with uracil−DNA glycosylase (Ung) as previously described (*16*) to convert the uracil site into an AP site.

Oligonucleotides containing a single strand gap with 5′- and 3′-hydroxy termini (SS-gap) at defined sites opposite to DHT on the complementary strand were prepared by hybridization of strand 1, described above for hybridization to uracil-containing oligonucleotides, with oligonucleotides 2a and 2b. The following oligonucleotides, 2a and 2b, have been used to vary the position of the gap relative to that of DHT: position −5, 2a, 5′-CTCTTAGTCAGGAA-3′OH, and 2b, 5′OH-ATGTATCTATGCTGGGAGCAAAGGC-3′; position −3, 2a, 5′-CTCTTAGTCAGGAATA-3′OH, and 2b, 5′OH-GTATCTATGCTGGGAGCAAAGGC-3′; position −1, 2a, 5′-CTCTTAGTCAGGAATATG-3′OH, and 2b, 5′OH-ATCTATGCTGGGAGCAAAGGC-3′; position +1, 2a, 5′-CTCTTAGTCAGGAATATGTA-3′OH, and 2b, 5′OH-CTATGCTGGGAGCAAAGGC-3′; position +3, 2a, 5′-CTCTTAGTCAGGAATATGTATC-3′OH, and 2b, 5′OH-ATGCTGGGAGCAAAGGC-3′; position +5, 2a, 5′-CTCTTAGTCAGGAATATGTATCTA-3′OH, and 2b, 5′OH-GCTGGGAGCAAAGGC-3′; position +8, 2a, 5′-CTCTTAGTCAGGAATATGTATCTATGC-3′OH, and 2b, 5′OH-GGGAGCAAAGGC-3′.

Oligonucleotides, containing 8-oxoG at defined positions opposite to a second 8-oxoG on the complementary strand, were prepared by hybridization of strand 1, containing 8-oxoG at a fixed position in the following sequence, 5′-GCCTTTGCTCCCAGCCTCGC(8-oxoG)CACTCTTCCTGACTAAGAG-3′, with strand 2 containing either 8-oxoG or guanine, as control, at the various defined positions opposite to 8-oxoG on strand 1: control, 5′-CTCTTAGTCAGGAAGAGTGCGCGAGGCTGGGAGCAAAGGC-3′; position −3, 5′-CTCTTAGTCAGGAAGA(8-oxoG)TGCGCGAGGCTGGGAGCAAAGGC-3′; position −1, 5′-CTCTTAGTCAGGAAGAGT(8-oxoG)CGCGAGGCTGGGAGCAAAGGC-3′; position +1, 5′-CTCTTAGTCAGGAAGAGTGC(8-oxoG)CGAGGCTGGGAGCAAAGGC-3′; position +3, 5′-CTCTTAGTCAGGAAGAGTGCGC(8-oxoG)AGGCTGGGAGCAAAGGC-3′.

Oligonucleotides, containing an AP site at defined positions relative to 8-oxoG on the complementary strand, were prepared by hybridization of strand 1, containing 8-oxoG at a fixed position in the following sequence, 5′-GCCTTTGCTCCCAGCATAGA(8-oxoG)ACATATTCCTGACTAAGAG-3′, with strand 2 containing either uracil or thymine, as control, at the various defined positions: control, 5′-CTCTTAGTCAGGAATATGTCTCTATGCTGGGAGCAAAGGC-3′; position −5, 5′-CTCTTAGTCAGGAAUATGTCTC-

TATGCTGGGAGCAAAGGC-3′; position −3, 5′-CTCTTAGTCAGGAATAUGTCTCTATGCTGGGAGCAAAGGC-3′; position −1, 5′-CTCTTAGTCAGGAATATGUCTCTATGCTGGGAGCAAAGGC-3′; position +1, 5′-CTCTTAGTCAGGAATATGTCUCTATGCTGGGAGCAAAGGC-3′; position +3, 5′-CTCTTAGTCAGGAATATGTCTCUATGCTGGGAGCAAAGGC-3′; position +, 5′-CTCTTAGTCAGGAATATGTCTCTAUGCTGGGAGCAAAGGC-3′.

Oligonucleotides containing a reduced AP site were prepared by mixing equal volumes of a solution of AP site/8-oxoG-containing double-stranded oligonucleotides described above with freshly prepared, 2 M solutions of $NaBH_4$ (Sigma). The resulting solution was incubated for 30 min at room temperature as described (*24*). The resulting oligonucleotide was then desalted by passage through a microspin G-25 column (Amersham Pharmacia Biotech) and precipitated with cold ethanol. The efficiency of reduction of the AP site was checked by labeling both strands of the oligonucleotide prior to incubation with Fpg protein, which does not cleave effectively a reduced AP site compared with its ability to cleave a nonreduced AP site, as control.

To obtain a single strand break with 5′-phosphate and 3′-phosphoaldehyde termini ($\beta$-SSB) at the various defined positions opposite to 8-oxoG on the complementary strand, the AP site/8-oxoG-containing oligonucleotides described above were treated with 10 ng of Nth protein as described in (*16*, *25*). The oligonucleotides were then precipitated with cold ethanol, washed, dried, and resuspended in the appropriate amount of TE buffer. The optimal amount of Nth protein required to nick all of the AP sites was determined from the migration profiles following PAGE of the labeled oligonucleotides which had been treated with varying amounts of Nth.

A single strand break with 5′-deoxyribose phosphate and 3′-OH termini (HAP1-SSB) (*26*) was obtained at the various positions relative to 8-oxoG on the complementary strand by treatment of AP site/8-oxoG-containing oligonucleotides with 500 pg of human AP endonuclease 1 (HAP1) in 50 $\mu$L of buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 1 mM $MgCl_2$, 20% glycerol) for 1 h at 37 °C. The oligonucleotides were then precipitated with cold ethanol, washed, dried, and resuspended in the appropriate amount of TE buffer. Under these conditions, the efficiency for formation of a HAP1-induced SSB is >98% (data not shown), as verified from the migration profiles of the labeled oligonucleotides following PAGE.

A single strand break with 5′- and 3′-phosphate termini ($\beta-\delta$-SSB) was obtained at various positions opposite to 8-oxoG on the complementary strand by hybridization of strand 1, containing the 8-oxoG at a fixed position in the following sequence, 5′-GCCTTTGCTCCCAGCATAGA(8-oxoG)ACATATTCCTGACTAAGAG-3′, with oligonucleotides 2a and 2b. The following strands (2a and 2b) have been used to vary the position of the gap relative to that of 8-oxoG: position −5, 2a, 5′-CTCTTAGTCAGGAA-3′P, and 2b, 5′P-ATGTCTCTATGCTGGGAGCAAAGGC-3′; position −3, 2a, 5′-CTCTTAGTCAGGAATA-3′P, and 2b, 5′P-GTCTCTATGCTGGGAGCAAAGGC-3′; position −1, 2a, 5′-CTCTTAGTCAGGAATATG-3′P, and 2b, 5′P-CTCTATGCTGGGAGCAAAGGC-3′; position +1, 2a, 5′-CTCTTAGTCAGGAATATGTC-3′P, and 2b, 5′P-CTATGCTGGGAGCAAAGGC-3′; position +3, 2a, 5′-CTCTTAGTCAGGAATATGTCTC-3′P, and 2b, 5′P-ATGCTGGGAGCAAAGGC-3′; position +5, 2a, 5′-CTCTTAGTCAGGAATATGTCTCTA-3′P, and 2b, 5′P-GCTGGGAGCAAAGGC-3′.

*Purified Proteins*. The purified Nth and HAP1 proteins were generous gifts from Prof. Rick Wood (ICRF) (*27*) and Dr. Ian Hickson (Institute of Molecular Medicine) (*26*), respectively. The purified Fpg protein was extracted and purified as described by Boiteux et al. (*28*).

*Cleavage Assays for SSB Analysis*. Known concentrations of double-stranded oligonucleotides (0.2−1 nM) were incubated with known amounts of Nth or Fpg proteins, as specified in the legends of the figures, for 1 h and 30 min, respectively, in 5 $\mu$L of the incubation buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA) at 37 °C. Subsequently, 5 $\mu$L of the denaturing stop solution was added (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 2 mM EDTA, pH 8.0) to the samples, which were then subjected to electrophoresis in a 12% denaturing polyacrylamide gel containing 8 M urea in 1× TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) for 90 min at 85 W. The dried gel was then exposed to a Bio-Rad PhosphorImager screen to visualize the cleaved and full-length DNA fragments using phosphorimagery (Bio-Rad, Molecular Imager FX) and quantified using Quantity One software (Bio-Rad) to determine the excision efficiency of each enzyme for each of the DNA sequences used. The number of nicks (cleaved strands) is a measure of the number of modified bases excised. The activity of the labeled, cleaved strand is expressed as a percentage of the total activity of the cleaved and intact strands. The efficiencies for excision of a base lesion within clustered damages are compared with that for excision of a single lesion in the control oligonucleotide at the same concentration of protein to assess the effect of the second lesion, present on the unlabeled strand, on excision of the lesion on the labeled strand. The errors represent standard errors of the mean from at least three repeat experiments.

*Kinetic Measurements*. For kinetic measurements, known concentrations of the oligonucleotides (0.25−100 nM) were incubated for 5 min at 37 °C with known concentrations of either Nth or Fpg protein, as specified in the figure legends or table. The concentration of the cleaved, labeled strand relative to that of the intact strand was determined as described above for SSB assay with denaturing PAGE. The initial reaction rate, obtained from the concentration of cleaved DNA per minute for a constant concentration of protein, was plotted versus the substrate concentration using a double reciprocal dependence based on Michaelis−Menten kinetics. Each point represents the standard error of the mean for at least three repeated experiments using six to eight different concentrations of the substrates. The values of $K_M$ and $V_{max}$ were obtained from the linear regression analysis of the Lineweaver−Burk plots, using the method of weighted least squares, the ordinate of each point being weighted by its reciprocal.

*Electromoblility Shift Assays (EMSA)*. To assess the ability of Fpg protein to bind to the oligonucleotides containing 8-oxoG, binding reactions were performed by mixing 5′-$^{32}$P-end-labeled oligonucleotides (10 000 cpm, 0.5 nM) with 10 $\mu$L of binding buffer (20 mM HEPES, pH 7.9, 100 mM in KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 0.2 mM

EDTA, 20% glycerol) or with 10 $\mu$L of buffer containing various amounts of nonlabeled, competitor oligonucleotide. The resulting solution was incubated for 20 min with Fpg protein (1 ng) at 0 °C. The samples were then loaded directly onto a 5% polyacrylamide gel as described in ref *29*. After electrophoresis at 35 mA in TAE 0.7× (4.7 mM Tris-HCl, 2.3 mM sodium acetate, 0.7 mM EDTA, pH 7.9) for 2 h, the gels were dried, exposed to a phosphorimager screen, and quantified as described above. The absence of PMSF, DTT, and $MgCl_2$ from the binding buffers did not influence the observations on the competitive binding of Fpg to the oligonucleotides (data not shown).

## RESULTS

Various double-stranded oligonucleotides, containing either a single DHT or 8-oxoG modification on the labeled strand positioned five, three, or one base 5′ or one, three, five, or eight bases 3′ to either another lesion (8-oxoA as a base lesion, an AP site, or a nucleotide gap on the complementary strand) or the normal corresponding base on the complementary strand, were used to determine the kinetics of excision of the base lesion on the labeled strand by either Nth or Fpg proteins. The concentrations of the proteins were varied to excise between 2% and 20% of the substrates from the total DNA to avoid either background effects at the lower level using the higher concentrations of DNA substrate or saturation effects at the lower concentrations of DNA substrate. The kinetic constants were obtained from Lineweaver−Burk plots, an example of which is shown in Figure 1S (see Supporting Information) for excision of DHT by Nth when either 8-oxoA or adenine, as control, was positioned one base 5′ to the site opposite to DHT (position +1). The variation of $K_M$, $k_{cat}$, and the specificity constants for excision of DHT by Nth relative to the positions of 8-oxoA/adenine is presented in Figure 1. The presence of an 8-oxoA opposite to DHT has little (position +1) or no effect (positions −3 and +3) on the kinetics of excision of DHT by Nth, when compared with the kinetic constants for the corresponding control.

The variation of the catalytic constants for cleavage of DHT by Nth in the presence of an AP site on the complementary strand one, three, or five bases 3′ or one, three, five, or eight bases 5′ to the site opposite to DHT is presented in Figure 1. The presence of an AP site significantly reduces the specificity constant for excision of DHT by Nth, compared with that of the control. This inhibitory effect reflects a large reduction in $k_{cat}$ whereas $K_M$ is only slightly affected. Since Nth protein also cleaves an AP site to give a SSB, the presence of a SS-gap on the efficiency of excision of DHT by Nth was examined. As shown in Figure 2, a SS-gap five or three bases 3′ to the site opposite to DHT only slightly inhibits the excision of DHT by Nth, whereas the inhibitory effect is greater when a SS-gap is positioned one base 3′ or 5′ to the site opposite to DHT. The kinetic constants for excision of DHT opposite to a SS-gap by Nth as shown in Figure 1 also reflect this inhibitory effect. Indeed, the specificity constant is reduced by up to 2 orders of magnitude when compared with that for control DNA, which contains DHT as a single lesion.

As shown in Figure 3, the effect of 8-oxoA on the specificity constants for the excision of DHT positioned three

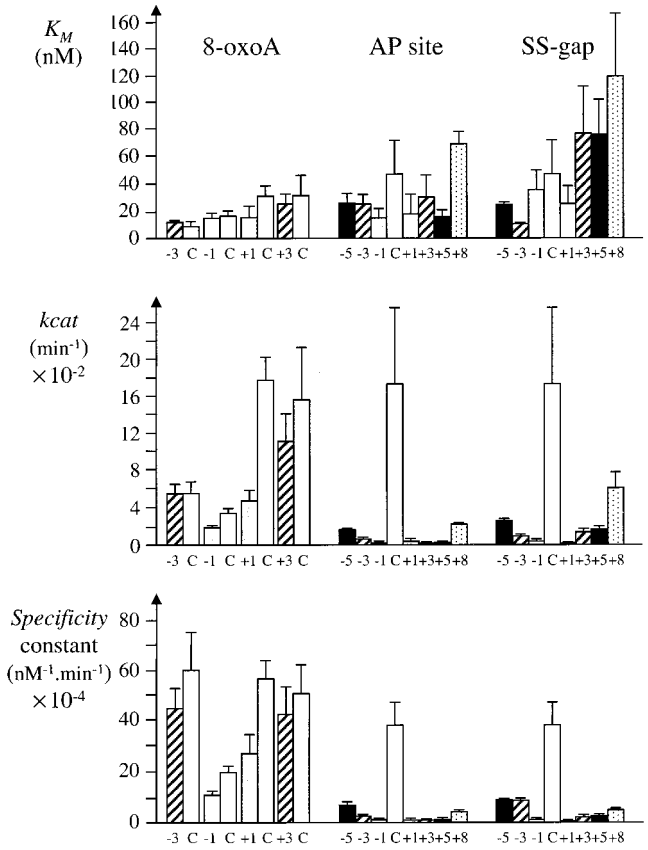


FIGURE 1: Variation of $K_M$, $k_{cat}$, and the specificity constants for cleavage of DHT by Nth (2.5 ng) on the interlesion separation with either 8-oxoA, an AP site, a SS-gap, or the normal base (C) on the nonlabeled, complementary strand of the oligonucleotides (1−30 nM).

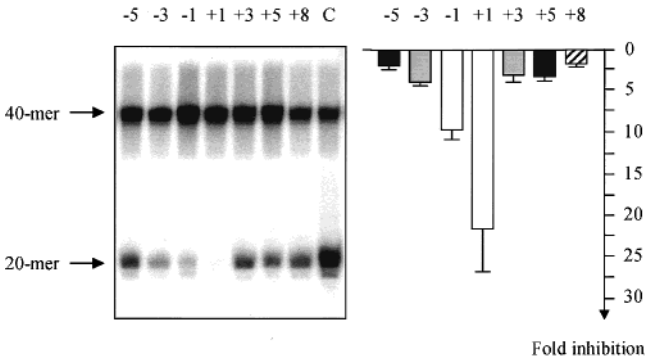


FIGURE 2: Effect of the presence of a SS-gap at various, defined positions on the nonlabeled strand on the efficiency of Nth (2.5 ng) to excise DHT at position 20 on the labeled strand, expressed as fold inhibition by comparison with the control containing a DHT. The percentage of DHT excised was measured after separation of the 20-mer from the 40-mer by denaturing PAGE.

bases 3′ or one and three bases 5′ to 8-oxoA by Fpg protein is similar to that for control DNA containing a single DHT at the various positions opposite to adenine instead of 8-oxoA. In contrast, the specificity constant for excision of a DHT positioned one base 3′ to the site opposite to 8-oxoA by Fpg protein (position −1, Figure 3) is greater (by a factor of 8) than that for the control DNA (position −1 (C), Figure 3). This activation is associated with a 10-fold decrease of the value of $K_M$ when compared with that of the control, whereas the value of $k_{cat}$ is similar. Using oligonucleotides containing an AP site or SS-gap at various positions opposite

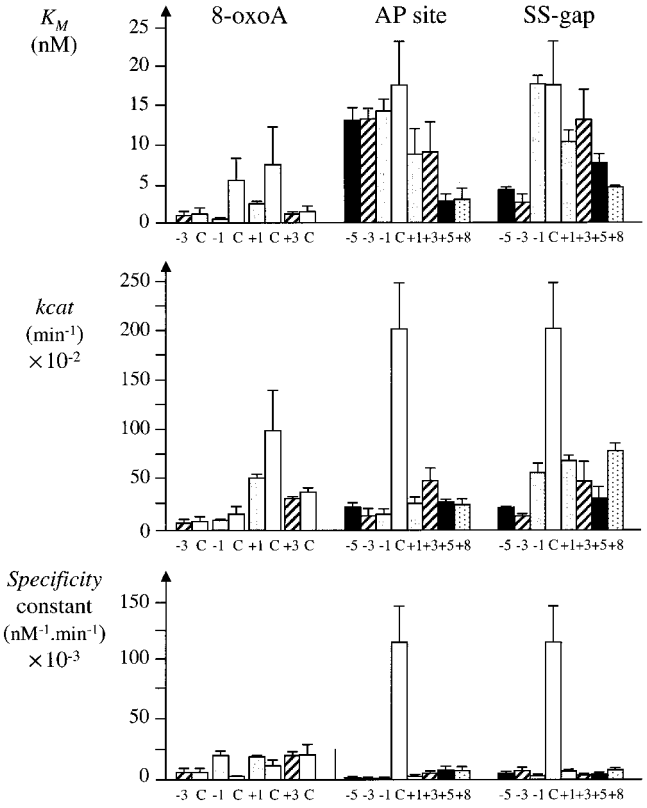


FIGURE 3: Variation of $K_M$, $k_{cat}$, and the specificity constants for cleavage of DHT by Fpg (1 ng with 8-oxoA and control oligonucleotides, otherwise 25 ng) on the interlesion separation with either 8-oxoA, an AP site, a SS-gap, or the normal base (C) on the nonlabeled, complementary strand of the oligonucleotides (0.25−50 nM).

to DHT, a large reduction in the specificity constants of up to 100-fold was observed for excision of DHT by Fpg protein, when compared with that of the control (Figure 3). The largest effect of an AP site occurs when at position −3 to DHT, as previously reported (*16*). Whereas the values of $K_M$ determined for the various oligonucleotides are of the same order of magnitude, the values of $k_{cat}$ are greatly reduced for excision of DHT in the presence of either an AP site or a SS-gap at all of the positions tested (Figure 3). The inhibitory effect, seen even when a SS-gap is at a significant distance from DHT (Figure 2S), extends over large interlesion distances for both Fpg and Nth proteins (Figures 1 and 3).

Since 8-oxoG is a biologically significant substrate for Fpg protein, the following experiments were undertaken. The dependence of the extent of excision of 8-oxoG, on the labeled strand, by Fpg protein on the relative positions of an additional 8-oxoG on the nonlabeled strand is shown in Figure 4A. The kinetic constants determined for excision of 8-oxoG in the presence of 8-oxoG on the complementary strand by Fpg protein are presented in Table 1. The major effects are seen when 8-oxoG is positioned one base 5′ or 3′ to the site opposite to 8-oxoG. In contrast, DHT situated one base 3′ or 5′ to the site opposite to 8-oxoG on the complementary strand causes a small increase in the efficiency of excision of 8-oxoG by Fpg protein. DHT at the other positions tested does not have a significant effect (Figure 3S). It is worth noting that for the corresponding oligonucleotides labeled on the strand containing DHT, the

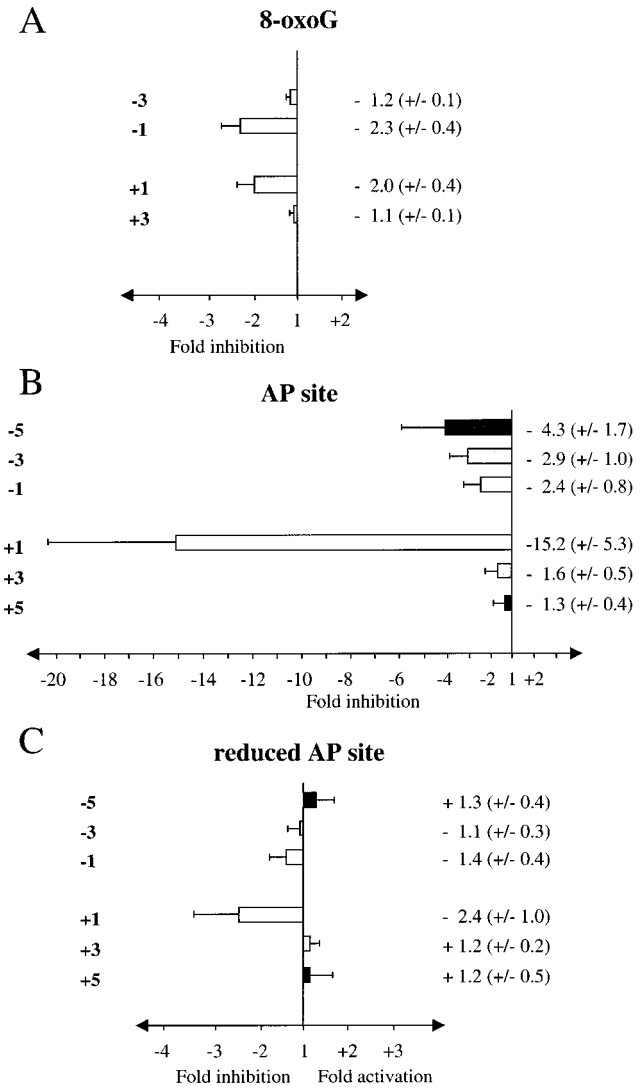


FIGURE 4: Effect of the presence of 8-oxoG, an AP site, or the normal base (C) at various, defined positions on the nonlabeled strand on the efficiency of Fpg (165−660 pM) to excise 8-oxoG on the labeled strand (1 nM), expressed as fold inhibition by comparison with the control containing an 8-oxoG.

extent of its excision by Fpg protein is reduced in the presence of an 8-oxoG up to three bases 5′ or 3′ to the site opposite to DHT (*16*).

The dependence of the extent of excision of 8-oxoG, on the labeled strand, by Fpg protein on the relative positions of an AP site on the nonlabeled strand is shown in Figure 4B. An inhibitory effect on excision of 8-oxoG occurs, especially when an AP site is situated one base 3′ to the site opposite to the 8-oxoG (position +1). Using uracil-containing oligonucleotides as controls, the presence of uracil does not influence the extent of excision of 8-oxoG by Fpg protein (Figure 4S). Since an AP site is removed more efficiently than 8-oxoG by Fpg protein from oligonucleotides containing either as a single lesion (Figure 5S), the inhibitory effect of an AP site on excision of 8-oxoG by Fpg protein may be due to rapid conversion of the AP site into a SSB by the Fpg protein. To address whether a SSB has an inhibitory effect on the excision of 8-oxoG by Fpg protein, a variety of oligonucleotides were constructed containing either a reduced AP site (refractory to excision by AP lyase proteins), a $\beta$-SSB, a HAP1-SSB, or a $\beta-\delta$-SSB (representing an Fpg-

Table 1: Kinetic Constants for the Excision of 8-OxoG by Fpg in the Presence of a Neighboring 8-OxoG or $\beta-\delta$-SSB on the Complementary Strand Determined from Lineweaver−Burk Plots[a]

| damage opposite to 8-oxoG | [S] (nM) | position | $K_M$ (nM) | $k_{cat}$ ($min^{-1}$) $\times 10^{-4}$ | specificity constant ($min^{-1}$·$nM^{-1}$) $\times 10^{-5}$ |
|---|---|---|---|---|---|
| 8-oxoG | 1.25−100 | −3 | 4.7 ± 1.4 | 1480 ± 130 | 3160 ± 800 |
| | | −1 | 6.4 ± 2.3 | 1180 ± 130 | 1850 ± 550 |
| | | +1 | 10.4 ± 2.1 | 1770 ± 130 | 1700 ± 240 |
| | | +3 | 4.9 ± 1.9 | 2020 ± 240 | 4110 ± 1330 |
| | | control | 6.6 ± 1.9 | 3180 ± 260 | 4820 ± 1140 |
| $\beta-\beta$-SSB | 1−50 | −5 | 20.0 ± 8.5 | 3210 ± 720 | 1610 ± 350 |
| | | −3 | 15.7 ± 2.8 | 1750 ± 130 | 1120 ± 130 |
| | | −1 | 28.7 ± 12.5 | 680 ± 180 | 240 ± 120 |
| | | +1 | 18.5 ± 8.9 | 430 ± 80 | 230 ± 70 |
| | | +3 | 18.6 ± 6.3 | 3040 ± 430 | 1630 ± 360 |
| | | +5 | 6.1 ± 1.4 | 2120 ± 140 | 3470 ± 610 |
| | | control | 7.2 ± 3.3 | 2680 ± 660 | 3730 ± 1140 |

[a] The oligonucleotides with 8-oxoG on the nonlabeled strand and the relative control DNA were incubated with 50 pg (0.33 nM), the oligonucleotides containing a $\beta-\delta$-gap at positions −1 and +1 with 500 pg (3.33 nM), and all other oligonucleotides with 100 pg (0.66 nM) of Fpg protein.

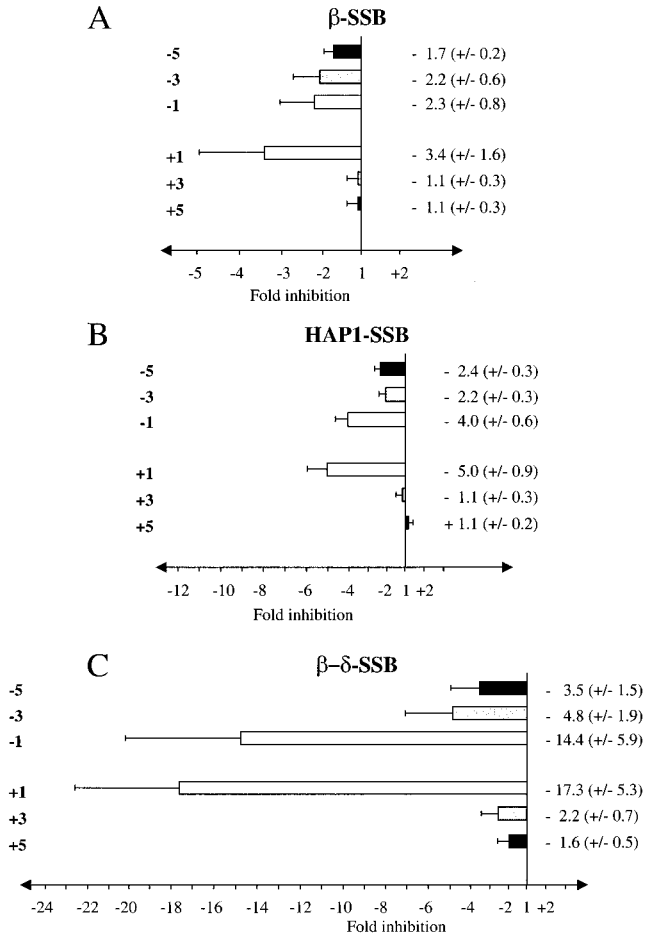


FIGURE 5: Effect of different types of single strand breaks, a $\beta$-SSB (A), an HAP1-SSB (B), or a $\beta-\delta$-SSB (C), on the efficiency of excision of 8-oxoG by Fpg protein (165−660 pM), expressed as fold inhibition by comparison with the control containing an 8-oxoG lesion.

induced SSB) on the nonlabeled strand at various, defined positions relative to 8-oxoG on the labeled strand. These various types of SSBs with different strand break termini are used as models of those induced in DNA by reactive oxygen species and ionizing radiation. A reduced AP site at the various positions studied has a significantly lower inhibitory effect (Figure 4C) than that of an AP site (Figure 4B), relative to that of the control, on the excision of 8-oxoG by Fpg protein.

Both a $\beta$-SSB and a HAP1-SSB reduce the extent of excision of 8-oxoG by Fpg protein, with the maximum inhibitory effect occurring when these SSBs are one base 3′ to the site opposite to 8-oxoG (position +1, Figure 5A,B). The extent of inhibition with these SSBs is larger than that seen with a reduced AP site (Figure 4C). Of the SSBs studied, the largest inhibitory effect on the excision of 8-oxoG by Fpg protein, when compared with the extent of excision of the control, is seen with a $\beta-\delta$-SSB (Figure 5C). The extent of inhibition and the interlesion distance over which inhibition occurs are similar to those seen in the presence of an AP site (Figure 4B). The kinetic constants for excision of 8-oxoG within clustered damage containing a $\beta-\delta$-SSB by Fpg protein are presented in Table 1. The specificity constants decrease by a factor of 2−3 relative to that of the control DNA, if a $\beta-\beta$-SSB is present at positions −5, −3, and +3. However, a decrease in the specificity constant of greater than one order of magnitude was observed for excision of 8-oxoG by Fpg protein when a $\beta-\delta$-SSB is positioned one base 3′ or 5′ to the site opposite to 8-oxoG. Only small changes in the values of $K_M$ were observed, whereas a large decrease in the value of $k_{cat}$ relative to that of the control occurs when a $\beta-\delta$-SSB is present one base 3′ or 5′ to the site opposite to 8-oxoG, consistent with the large decrease of the enzyme activity (Figure 5C).

Since the values of $K_M$ for excision of either DHT or 8-oxoG in all DNA samples are only slightly modified by a base lesion on the complementary strand, whereas the values of $k_{cat}$ are greatly affected, EMSAs were performed to determine if the recognition of the substrate by the proteins is influenced by the presence of a lesion on the complementary strand. With oligonucleotides containing DHT opposite to an AP site or a SS-gap, the extent of binding of either Nth or Fpg proteins to these oligonucleotides at 4 °C is very weak, even when large amounts of protein (up to 0.5 $\mu$g) were used. No significant gel shift was observed for the other oligonucleotides containing DHT opposite to an AP site or a SSB and Nth. Band shifts were however observed corresponding to binding of Fpg to oligonucleotides containing 8-oxoG opposite to either 8-oxoG, a $\beta-\delta$-SSB, or the normal base at various positions on the complementary strand. From incubation of the various oligonucleotides with increasing amounts of Fpg protein, the apparent binding constants of Fpg protein to oligonucleotides containing a

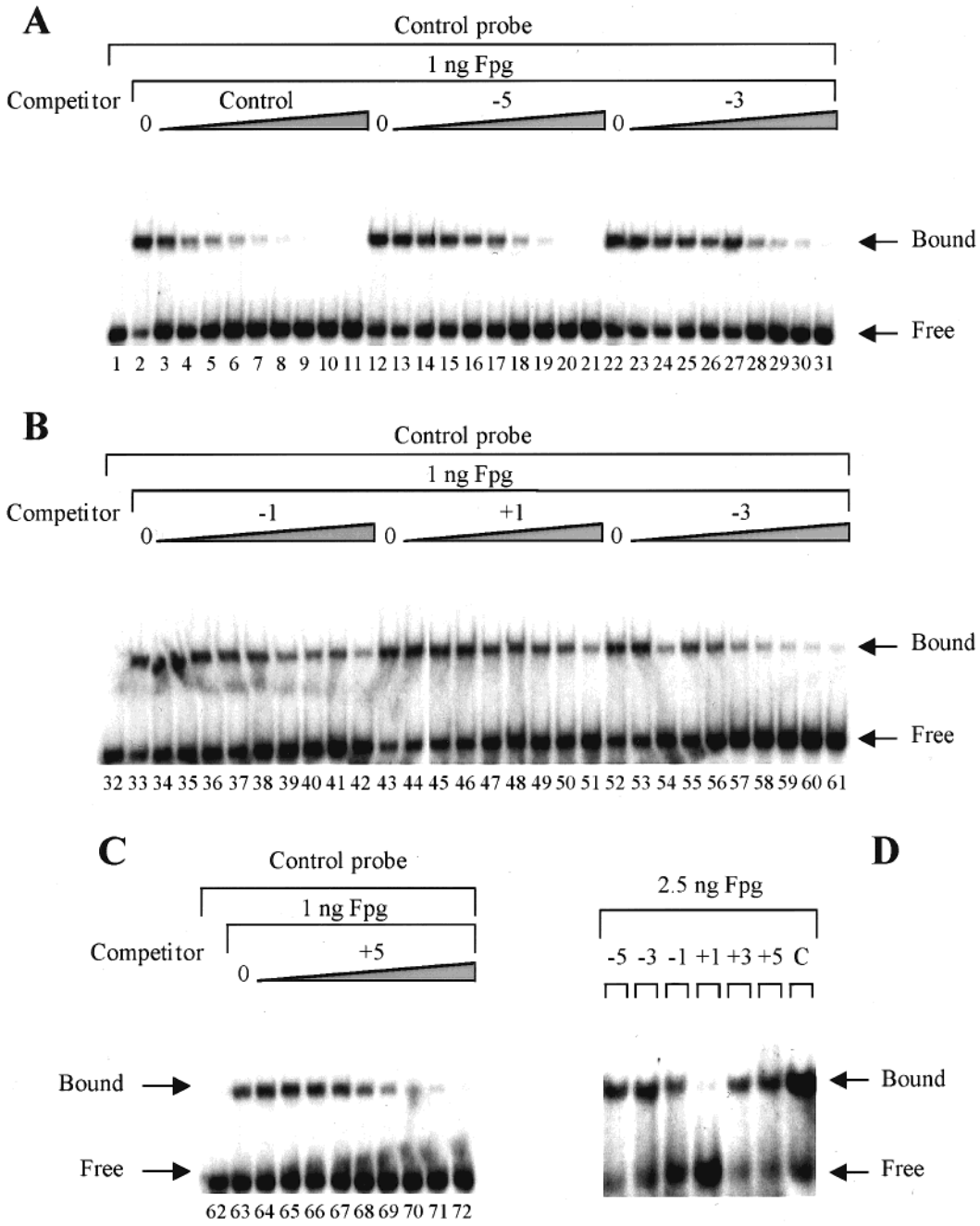


FIGURE 6: (A−C) Gel profiles using EMSA showing the binding of Fpg protein (3.3 nM) to a $^{32}$P-labeled oligonucleotide containing 8-oxoG as a single lesion (control probe, 0.5 nM) in the absence (lanes 1, 32, and 62, buffer only; lanes 2, 12, 22, 33, 43, 52, and 63, Fpg and buffer) or the presence of increasing amounts of nonlabeled oligonucleotides, as competitor, containing either 8-oxoG as a single damage (control) or an 8-oxoG opposite to a $\beta-\delta$-gap at the various, defined positions. The quantity of competitor oligonucleotide compared with that of the labeled control oligonucleotide are as follows: 5×, lanes 3, 13, 23, 34, 44, 53, 64; 10×, lanes 4, 14, 24, 35, 54, 65; 20×, lanes 5, 15, 25, 36, 45, 55, 66; 40×, lanes 6, 16, 26, 37, 46, 56, 67; 80×, 7, 17, 27, 38, 47, 57, 68; 160×, lanes 8, 18, 28, 39, 48, 58, 69; 320×, lanes 9, 19, 29, 40, 49, 59, 70; 640×, lanes 10, 20, 30, 41, 50, 60, 71; and 1280×, 11, 21, 31, 42, 51, 61, 72. (D) Gel retardation assay with Fpg protein (2.5 ng) and 0.5 nM probe containing 8-oxoG as a single damage (C) or opposite to a $\beta-\beta$-gap at the positions defined.

single 8-oxoG or two opposite 8-oxoG were determined to be ~2−3 nM (data not shown). As shown in Figure 6 (panel D), incubation of the various oligonucleotides containing a $\beta-\delta$-SSB opposite to 8-oxoG at 4 °C with Fpg protein results in a band shift that becomes less intense as the interlesion separation between 8-oxoG and the $\beta-\delta$-SSB decreases. The largest extent of inhibition on binding of Fpg to 8-oxoG occurs when a $\beta-\beta$-SSB is at position +1. The relative affinities of binding of Fpg protein to the various oligonucleotides were estimated from the level of Fpg bound to the labeled, double-stranded oligonucleotide, which contains 8-oxoG as a single lesion, in the presence of increasing amounts of either nonlabeled control oligonucleotide (C) or nonlabeled oligonucleotides which also contained a $\beta-\delta$-SSB (Figure 6, panels A−C). The amount of the Fpg/control DNA complex, estimated from the intensity of the shifted band, is reduced in the presence of increasing amounts of competitor oligonucleotide as control (lanes 3−11) or oligonucleotides containing a $\beta-\delta$-SSB five or three bases 3′ or 5′ to the site opposite to 8-oxoG (lanes 13−21, 23−

31, 53−61, and 64−72, respectively). For oligonucleotides containing a $\beta-\delta$-SSB one base 3′ or 5′ to the site opposite to 8-oxoG (lanes 44−51 and 53−61, respectively) binding to the labeled oligonucleotide is only weakly reduced by the competitor oligonucleotides. It is estimated that the binding coefficient of Fpg to 8-oxoG when one base 5′ or 3′ to the site opposite to a $\beta-\delta$-SSB is about 1 order of magnitude less than that of the control.

## DISCUSSION

From comparison of the specificity constants, it is clearly demonstrated that either an AP site or various types of SSBs within a clustered DNA damage have large inhibitory effects, when compared with the control, on the excision of either DHT by Nth or Fpg proteins or 8-oxoG by Fpg protein, when present within a few base pairs on the complementary strand. The main effect of the presence of these lesions (an AP site or various SSBs) on the excision of DHT by Fpg or Nth protein is a decrease of up to 2 orders of magnitude in the values of $k_{cat}$, whereas $K_M$ is only slightly modified, when compared with the corresponding values for control DNA containing a single DHT. Since the glycosylase activities of Fpg and Nth proteins are significantly less than their corresponding AP lyase activities (*16*), it is assumed that the rate-determining step for formation of a SSB on excision of the DNA base lesions is the glycosylase activities of the proteins. The influence of a SS-gap on $k_{cat}$ for removal of 8-oxoG by Fpg protein is significantly less than the corresponding values for removal of DHT by Nth or Fpg proteins. In contrast to the inhibitory effects of various SSBs and an AP site, the values of $K_M$ and $k_{cat}$ and the specificity constants for excision of either DHT by Nth or Fpg or 8-oxoG by Fpg protein are not significantly different from that of the control even when a base lesion (8-oxoA or 8-oxoG) is only one base pair away on the complementary strand. The specificity constants, and the values of $K_M$ and $k_{cat}$, determined for excision of a single 8-oxoG lesion by Fpg protein when complementary to cytosine are similar to the values determined previously (*20*, *23*). With Nth, the values of $k_{cat}$ for excision of a single DHT lesion complementary to an adenine base are similar to those determined previously, whereas the value of $K_M$ is a factor of 10 less than those previously reported (*30*, *31*).

Differences in the inhibitory effects of the various types of SSBs on the excision of 8-oxoG within a clustered damage by Fpg may reflect differences in the end termini of the SSB. The inhibitory effect seen with a $\beta-\delta$-SSB, which has 3′- and 5′-phosphate termini, is significantly larger than that with either a HAP1-SSB or a $\beta$-SSB, which have sugar remnants on the 5′- or 3′-terminus, respectively. Whether these differences reflect variations in the overall charge around the SSB and/or differences in minor structural perturbations around the site is as yet not known.

The extent of inhibition on excision of a base lesion within a clustered damage on the interlesion separation distance is consistent with those reported previously (*16*−*18*), based upon the yield of SSB formed by excision of base damage per unit time for a given concentration of enzyme. From the previously reported extent of inhibition observed in processing a base lesion when present within a clustered damage by BER proteins (*16*−*19*), it was not possible to assess whether the inhibitory effect on excision of a substrate when present within a clustered damage is due to reduced damage/protein binding (recognition), reduced rate of excision of the damaged base (glycosylase action), or a combination of both. The reduction in the binding affinity of Fpg protein to DNA containing 8-oxoG when in the presence of a $\beta-\delta$-SSB corresponds with the reduction of the specific activity of Fpg protein for excision of 8-oxoG, when a $\beta-\delta$-SSB is one base pair separated on the complementary strand. It is suggested that the reduction in specific activity reflects, in part, inhibition of binding of Fpg protein to 8-oxoG, when present in a clustered DNA damage containing a $\beta-\delta$-SSB at the various interlesion separations tested. The extend of binding of Fpg protein to a reduced AP site, which is refractory to incision by Fpg, is reduced when a SSB is one base pair from the reduced AP site. With Fpg and Nth proteins, binding to the oligonucleotides containing DHT was not detected as a band shift, since the majority of the DNA remained in the unbound form, even though a large excess of protein was added. Therefore, it is not possible to comment on whether the effect of a neighboring lesion influences the binding of Nth or Fpg proteins to DHT within DNA. However, the specificity constants and values of $k_{cat}$ for removal of DHT when present within a clustered damage by Nth is greatly reduced, even for interlesion distances of up to eight base pairs. It is suggested that the excision of DHT by Nth protein and not the binding (recognition) is significantly influenced by the proximity of a SSB on the complementary strand.

In summary, SSBs and an AP site but not base lesions significantly inhibit the overall efficiency of excision of DHT by Nth or Fpg proteins and of 8-oxoG by Fpg protein, when these lesions are present within a clustered damage. This inhibition is in part due to reduced binding (recognition) of Fpg to 8-oxoG and to a reduction in the kinetics of the catalytic excision step, particularly for DHT by Nth and Fpg proteins, when these base lesions are present within a clustered DNA damage. These findings are biologically very important since a reduced efficiency in excision of a damaged base within a clustered damage may allow sufficient time delay between the excision/repair processing of one lesion with respect to that of the other lesion. For instance, a SSB may be repaired before the excision/repair processing of a neighboring base lesion on the complementary strand is initiated. In this way the formation of highly toxic double strand breaks, in addition to those produced immediately by radiation, might be avoided in cells.

## ACKNOWLEDGMENT

We are grateful to Profs. Rick Wood (Imperial Cancer Research Fund) and Ian Hickson (Institute of Molecular Medicine) for providing the purified Nth and HAP1 proteins, respectively, and to Dr. David Papworth for assistance with the statistical analysis of the data.

## SUPPORTING INFORMATION AVAILABLE

Figure 1S showing Lineweaver−Burk plots for removal of DHT by Fpg, Figure 2S showing the effect of a SSB on the efficiency of excision of DHT by Fpg, Figures 3S and 4S showing the effect of DHT or uracil on the efficiency of excision of 8-oxoG by Fpg, respectively, and Figure 5S showing the dependence of the extent of excision of an AP

site or an 8-oxoG as a single damage on the [Fpg]. This material is available free of charge via the Internet at http://pubs.acs.org.

## REFERENCES


1. Goodhead, D. T., Thacker, J., and Cox, R. (1993) *Int. J. Radiat. Res. 63*, 543−556.
2. Ward, J. F. (1988) *Prog. Nucleic Acid Res. 35*, 95−125.
3. Nikjoo, H., O'Neill, P., Terrisol, M., and Goodhead, D. T. (1999) *Radiat. Environ. Biophys. 38*, 31−38.
4. Cunniffe, S., and O'Neill, P. (1999) *Radiat. Res. 152*, 421−427.
5. Sutherland, B. M., Bennett, P. V., Sidorkina, O., and Laval, J. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 103−108.
6. Laval, J., Jurado, J., Saparbaev, M., and Sodorkina, O. (1998) *Mutat. Res. 402*, 93−102.
7. Boiteux, S. (1993) *J. Photochem. Photobiol., B 19*, 87−96.
8. Demple, B., and Harrison, L. (1994) *Annu. Rev. Biochem. 63*, 915−948.
9. Demple, B., and Linn, S. (1980) *Nature 287*, 203−208.
10. Breimer, L. H., and Lindahl, T. (1984) *J. Biol. Chem. 259*, 5543−5548.
11. Dizdaroglu, M., Laval, J., and Boiteux, S. (1993) *Biochemistry 32*, 12105−12111.
12. Hatahet, Z., Kow, Y. W., Purmal, A. A., Cunningham, R. P., and Wallace, S. (1994) *J. Biol. Chem. 269*, 18814−18820.
13. Chetsanga, C. J., and Lindahl, T. (1979) *Nucleic Acids Res. 6*, 3673−3684.
14. Boiteux, S., Gajewski, E., Laval, J., and Dizdaroglu, M. (1992) *Biochemistry 31*, 106−110.
15. Tchou, J., Kasai, H., Shibutani, S., Chung, M.-H., Laval, J., Grollman, A. P., and Nishimura, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4690−4694.
16. David-Cordonnier, M. H., Laval, J., and O'Neill, P. (2000) *J. Biol. Chem. 275*, 11865−11873.
17. Harrison, L., Hatahet, Z., Purmal, A. A., and Wallace, S. S. (1998) *Nucleic Acids Res. 26*, 932−941.
18. Harrison, L., Hatahet, Z., and Wallace, S. S. (1999) *J. Mol. Biol. 290*, 667−684.
19. Chaudhry, M. A., and Weinfeld, M. (1997) *J. Biol. Chem. 272*, 15650−15655.
20. Tchou, J., Bodepudi, V., Shibutani, S., Antoshchkin, I., Miller, J., Grollman, A. P., and Johnson, F. (1994) *J. Biol. Chem. 269*, 15318−15324.
21. Karakaya, A., Jaruga, P., Bohr, V. A., Grollman, A. P., and Dizdaroglu, M.(1997) *Nucleic Acids Res. 25*, 474−479.
22. Sidorkina, O. M, and Laval, J. (1998) *Nucleic Acids Res. 26*, 5351−5357.
23. Jurado, J., Saparbaev, M., Matray, T. J., Greenberg, M. M., and Laval, J. (1998) *Biochemistry 37*, 7757−7763.
24. Castaing, B., Boiteux, S., and Zelwer, C. (1992) *Nucleic Acids Res. 20*, 389−394.
25. Bailly, V., and Verly, W. G. (1987) *Biochem. J. 242*, 565−572.
26. Barzilay, G., and Hickson, I. D. (1995) *BioEssays 17*, 713−719.
27. Asahara, H., Wistort, P. M., Bank, J. F., Bakerian, R. H., and Cunningham, R. P. (1989) *Biochemistry 28*, 4444−4449.
28. Boiteux, S., O'Connor, T. R., and Laval, J. (1987) *EMBO J. 6*, 3177−3183.
29. David-Cordonnier, M.-H., Hamdane, M., Bailly, C., and D'Halluin, J.-C. (1998) *Biochemistry 37*, 6065−6076.
30. D'Ham, C., Romieu, A., Jaquinod, M., Gasparutto, D., and Cadet, J. (1999) *Biochemistry 38*, 3335−3344.
31. Asagoshi, K., Odawara, H., Nakano, H., Miyano, T., Terato, H., Ohyama, Y., Seki, S., and Ide, H. (2000) *Biochemistry 39*, 11389−11398.


BI002605D