Enzymatic recognition of DNA modifications induced by singlet oxygen and photosensitizers

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ABSTRACT

DNA modifications induced either by photosensitization (illumination in the presence of methylene blue) or by chemically generated singlet oxygen (thermal decomposition of an 1,4-etheno-2,3-benzodioxin) are recognized and incised by repair endonucleases present in crude bacterial cell extracts. Only a small fraction of the incised modifications are sites of base loss (AP-sites) sensitive to exonuclease III, endonuclease IV from *E. coli* or to the UV-endonuclease from *M. luteus*. Cell extracts from *E. coli* strains overproducing or defective in endonuclease III recognize the modifications induced by illumination in the presence of methylene blue just as well as do those from wild-type *E. coli* strains. This indicates that dihydropyrimidine derivatives, which are characteristic of hydroxyl radical-induced DNA modifications, are absent. In contrast, most of the modifications induced are not recognized by a cell extract from a fpg strain defective in formamidopyrimidine-DNA glycosylase (FPG protein). Furthermore, incision by a cell extract from an *E. coli* strain overproducing FPG protein takes place at much lower protein concentration than with the wild-type strain. Experiments with purified FPG protein confirm that this enzyme is responsible for the recognition of singlet oxygen-induced DNA base modifications.

INTRODUCTION

Intracellular DNA damage caused by reactive oxygen species is known to be mutagenic and probably represents a major natural hazard for the genomic stability of living cells (1−3). This is demonstrated, for example, by the presence of specific repair enzymes for oxidative DNA damage in all types of cells investigated so far. The enzymes, which are typically glycosylases with an associated endonuclease activity for sites of base loss (AP sites), initiate repair independently of the nucleotide excision repair-systems of the cells (represented in *E. coli* by uvrABC)(4,5).

Among the reactive oxygen species that are likely to be generated inside cells, hydroxyl radicals (·OH) are the most reactive. Specific repair enzymes have been isolated (7,8) for many of the DNA modifications detected after exposure to hydroxyl radicals (6). Thus, endonuclease III in *E. coli* (9−11) and similar enzymes in other species, termed redoxyendonucleases or gamma-endonucleases (12), recognize various dihydropyrimidine derivatives, endonuclease IV can incise AP-sites oxidized in the sugar moiety (7,13,14), the FPG protein (formamidopyrimidine-DNA glycosylase) is known to recognize imidazole ring-opened purines (15−17) and a glycosylase specific for 5-hydroxymethyluracil is present in mammalian cells (18). The existence of all these enzymes is an indication that hydroxyl radicals are ultimate DNA modifying species in the cells under natural conditions.

Singlet oxygen is another reactive oxygen species which is formed under conditions of oxidative stress simultaneously with or in consequence of radical production (19−21). Furthermore, it is generated by photosensitizers such as riboflavin and many xenobiotics in the presence of light (22,23). Singlet oxygen most probably can react as an ultimate DNA modifying species inside cells (24) and give rise to mutagenic modifications (24−26). The chemical character of the DNA modifications, however, is not known. As singlet oxygen is not a radical, the modifications are expected to be different from those induced by hydroxyl radicals. Indeed, products of a (2+4) or (2+2) cycloaddition have been observed with guanosine *in vitro* (27).

In a previous study, we found that DNA modifications induced by singlet oxygen are recognized by repair endonucleases present in crude cell extracts of both *Salmonella typhimurium* and *Micrococcus luteus* (28). Here, we present a study on the character of the DNA modifications that are recognized and on the enzymes involved. We demonstrate that the endonuclease-sensitive modifications induced by singlet oxygen are modified base residues which are recognized by FPG protein.

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MATERIALS AND METHODS

DNA, bacterial strains, enzymes and chemicals

DNA from bacteriophage PM2 (PM2 DNA) was prepared according to the method of Salditt et al. (29). E. coli strains AB1157, AB1157/pFPG10 (overproducing FPG protein 12 fold) and BH20 (=AB1157 *fpg-1::kan*) have been described earlier (30,31). RPC51/pRPC53 is a transformant of RPC51 (32) overproducing endonuclease III 10–15 fold. The plasmid pRPC53 is a pBR322 derivative containing a 6.7 kb fragment from pLC9–9 which includes the *nth* gene (33). RPC37 (=AB1157 *nth-1::kan*) was constructed in the same fashion as BW372 (33). FPG protein was prepared as reported elsewhere (15). Endonuclease IV was kindly provided by Drs. J. Levin and B. Demple, Cambridge. Exonuclease III was obtained from Boehringer Mannheim, FRG. $NDPO_2$ (disodium salt of 1,4-etheno-2,3-benzodioxin-1,4-dipropanoic acid) was prepared as described by Nieuwint et al. (34).

Cell extracts and endonuclease preparations

Crude cell extracts from *E. coli* strains AB1157, RPC51/pRPC53 and RPC37 or AB1157, AB1157/pFPG10 and BH20 were prepared in parallel from overnight cultures (200 ml). Bacteria were pelleted, resuspended in 5 ml buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 15 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol) and disrupted by sonication. After addition of 100 mg streptomycin sulfate, stirring for 1 h at 4°C and centrifugation (15 min, 10,000 g) the supernatants were diluted in BE_{15} buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 15 mM EDTA) and stored in frozen aliquots.

A crude cell extract from *M. luteus* (ATCC 4698) was obtained as described by Riazzudin (35). A partially purified preparation of the UV-endonuclease from *M. luteus* was obtained after streptomycin sulfate and ammonium sulfate precipitations by diethylaminoethyl (DEAE)-cellulose chromatography. It was shown to fully recognize thymine dimers generated in PM2 DNA by UV^{260} (10 J/m^2) and not to incise thymine glycols in OsO_4-treated DNA.

Modification of PM2 DNA

PM2 DNA (10 μg/ml in phosphate buffer (5 mM NaH_2PO_4, 50 mM NaCl, pH 7.4) was exposed to one of the following conditions: (a) 4 sec visible light (Philips halogen lamp PF811 (1000 W) at a distance of 93 cm) in the presence of methylene blue (10 μg/ml) (b) 3 min pH 4.5 at 70°C to generate AP-sites as described by Lindahl and Nyberg (36) (c) 1 mM OsO_4 for 15 min at 24°C to generate thymine glycols according to the method of Demple et al. (37) (d) 3.5 mM $NDPO_2$ for 2 h at 37°C in a buffer in which H_2O has been substituted by D_2O. The PM2 DNA was precipitated (ethanol/ sodium acetate) and redissolved at 10 μg/ml in BE_1 buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA).

Incubation with endonucleases

0.2 μg modified PM2 DNA dissolved in 20 μl BE_1 buffer were incubated for 30 min with 10 μl of an endonuclease or a cell extract in BE_{15} buffer. In the case of exonuclease III, EDTA in the buffer was substituted by Ca^{2+} (15 mM). The reaction was stopped by addition of 3 μl 10% SDS. For the determination of directly produced strand breaks, the incubation was carried out without endonucleases.

Quantification of strand breaks and endonuclease-sensitive sites

To determine the average number of strand breaks per DNA molecule produced either directly by the damaging agent (ssb) or by the subsequent enzymatic incision of the endonuclease-sensitive sites (ess), the relative amounts of supercoiled (I) and open circular (II) forms were measured. For this purpose, they were separated by agarose gel electrophoresis and quantified by fluorescence scanning (Scanner FTR20, Sigma Instruments, Berlin) after ethidium bromide staining (0.5 μg/ml) as described previously (28). For the calculation, a statistical (Poisson) distribution of breaks was assumed and a correction factor of 1.4 was applied to account for the relatively lower fluorescence of the supercoiled form (I) compared to the open circular form (II) (38):

$$ssb + ess = -\ln [1.4*I / (1.4*I + II)]$$

For samples treated with endonucleases, the original values give the sum of single strand breaks (ssb) and endonuclease-sensitive sites (ess). When appropriate, the figures for the latter were obtained by subtraction. The calculated values for ssb and ess were corrected for those observed in control DNA not exposed to the damaging agent. These background values did not exceed 0.2 sites/10,000 bp for the highest concentrations of crude cell extracts.

RESULTS

DNA modifications induced by singlet oxygen are predominantly base modifications

For analysis of the DNA damage induced by singlet oxygen, we exposed supercoiled PM2 DNA (10,000 base pairs) in phosphate buffer to either $NDPO_2$, a chemical source of singlet oxygen (34,39), or to methylene blue in the presence of visible light. Previous studies have indicated that singlet oxygen is the

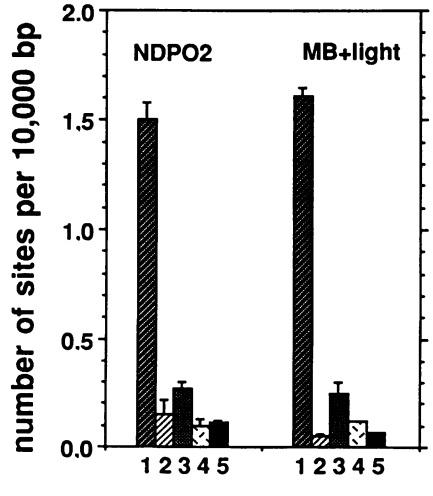

Figure 1. Single strand breaks and various endonuclease sensitive sites in PM2 DNA exposed to $NDPO_2$ (3.5 mM; 2h; 37°C; H_2O in the buffer replaced by D_2O) (left) or to methylene blue (10 μg/ml) in the presence of visible light (1000 W; 93 cm; 4 sec) (right). Columns (1–4) indicate the number of sites sensitive to (1) a crude endonuclease preparation from *M. luteus* (0.1 mg/ml protein), (2) exonuclease III (200 U/ml), (3) endonuclease IV (3 U/ml) and (4) a UV-endonuclease preparation from *M. luteus*. Columns (5) give the number of single strand breaks generated by the agents.

predominant ultimate DNA modifying species in both cases (28). After DNA modification we determined the number of endonuclease-sensitive sites recognized and incised by a crude protein preparation from *M. luteus* and the number of AP-sites detected by three different AP-endonucleases, viz. exonuclease III and endonuclease IV from *E. coli* and an UV-endonuclease preparation from *M. luteus*. In addition, the number of DNA single strand breaks generated by the agents was quantified.

The results shown in Fig. 1 indicate for both types of agent that sites sensitive to AP-endonucleases represent only a minor fraction of the total endonuclease-sensitive sites detected by the crude protein extract. Similarly, as observed earlier (28), strand breakage by the agents is low. As both regular AP-sites (generated by mere hydrolysis) and AP-sites oxidized in the sugar moiety most probably are recognized by at least one of the three AP-endonucleases (14), the majority of the DNA modifications induced by singlet oxygen are base modifications.

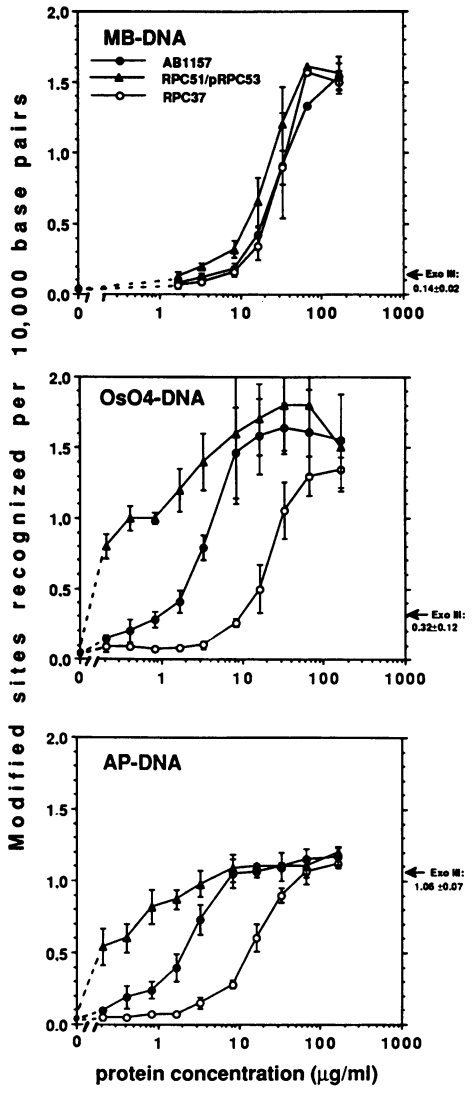

Figure 2. Recognition of modifications in PM2 DNA induced by methylene blue in the presence of light (upper panel), osmium tetroxide (central panel) and low pH (AP-DNA; lower panel) by crude protein extracts of *E. coli* strain AB1157 (●; 'wild-type'), RPC51/pRPC53 (▲; overproducing endonuclease III) and RPC 37 (○; defective in endonuclease III). Data points are means of three independent determinations (± S.D.) and represent the sum of endonuclease-sensitive sites and single strand breaks, i.e. values for the latter have not been subtracted and are represented by the data points given for zero protein concentration. For each type of agent, the recognition of AP-sites by high concentrations of exonuclease III (200 U/ml) is indicated by an arrow.

Endonuclease III is not involved in the recognition of singlet oxygen-induced DNA modifications

To test whether endonuclease III can recognize DNA base modifications induced by singlet oxygen, we compared the endonuclease activities present in protein extracts from a strain overproducing endonuclease III 10–15 fold (RPC51/pRPC53), an endonuclease III-deficient strain (RPC37) and a wild-type strain (AB1157) of *E. coli*.

The data shown in Fig. 2 indicate that the DNA modifications induced by methylene blue in the presence of light are recognized and incised equally well by extracts of all three strains. In contrast, DNA modifications induced by osmium tetroxide,

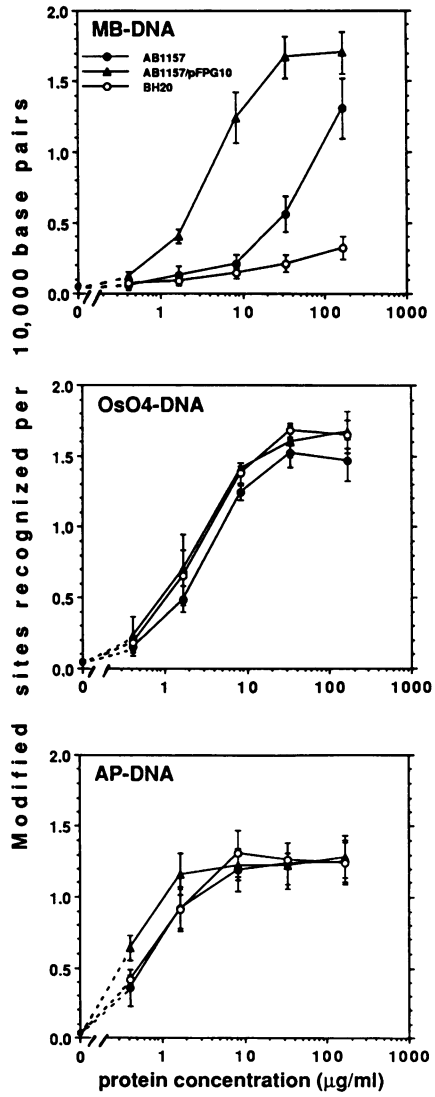

Figure 3. Recognition of modifications in PM2 DNA induced by methylene blue in the presence of light (upper panel), osmium tetroxide (central panel) and low pH (AP-DNA; lower panel) by crude protein extracts of *E. coli* strain AB1157 (●; 'wild-type'), AB1157/pFPG10 (▲; overproducing FPG protein) and BH20 (○; fpg). Experiments were carried out as for Fig. 2.

predominantly thymine glycols, are recognized at much lower protein concentrations by the extract from the overproducing strain than by that from the wild-type strain. The enzyme activity of the strain defective in endonuclease III is another order of magnitude lower than that of the wild-type strain. The fact that it is not zero indicates that endonuclease III is not the only enzyme of *E. coli* to recognize thymine glycols (40).

When DNA partly depurinated by incubation under acidic conditions (AP-DNA) is used as a target for the enzyme extracts from the three strains, the differences in the modification-incising activity are similar to those with the osmium tetroxide-treated DNA. Therefore, endonuclease III is a major component of the AP-endonuclease activity of the protein extracts under the conditions used.

In conclusion, endonuclease III does not contribute significantly to the recognition of singlet oxygen-induced base modifications by crude protein extracts from bacteria.

Endonuclease-sensitive base modifications induced by singlet oxygen are recognized by FPG protein

One of the few repair endonucleases known to recognize modified guanine residues in DNA is FPG protein, an enzyme shown to incise imidazole ring-opened purines (formamidopyrimidines)(16,41). To test whether FPG protein is involved in the recognition of base modifications induced by singlet oxygen, we have compared the abilities of crude cell extracts from the E. coli strain AB1157/FPG10 overproducing FPG protein 12-fold (30), the wild-type strain AB1157 and the FPG protein-defective strain BH20 (31) to incise these modifications.

The results shown in Fig. 3 indicate that DNA modifications induced by methylene blue in the presence of light are incised by a cell extract from FPG10 at much lower protein concentrations than by an extract from the corresponding wild-type strain AB1157; only very few sites are incised even at high protein concentrations by an extract from the defective strain BH20. On the other hand, no significant difference between the three strains is observed with DNA modified by osmium tetroxide (containing thymine glycols) or by low pH (containing regular AP-sites).

The recognition of singlet oxygen induced DNA base modifications by FPG protein is confirmed in experiments with the purified enzyme (Fig. 4). Modified sites generated by exposure to $NDPO_2$ and to methylene blue in the presence of light exhibit the same concentration dependence of the incision by the enzyme. In contrast, regular AP sites (generated by low pH) are incised by FPG protein at lower protein concentrations (Fig. 4). The frequency of incisions of osmium tetroxide-modified DNA is very low; even at high enzyme concentrations it does not exceed the level of AP-sites detected by exonuclease III (see Fig. 2).

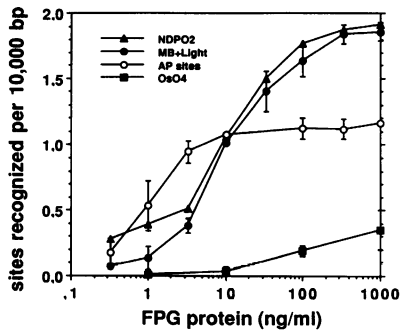

Figure 4. Recognition by purified FPG protein of modifications induced in PM2 DNA by exposure to (a) $NDPO_2$ (▲), (b) methylene blue in the presence of light (●), (c) osmium tetroxide (■) or (d) low pH (AP-sites; ○).

DISCUSSION

The results demonstrate that singlet oxygen generated either by thermal decomposition of $NDPO_2$ or by photosensitization with methylene blue gives rise to DNA base modifications which are recognized and incised by FPG protein. On the other hand, dihydropyrimidine derivatives, which are characteristic for damage induced by radicals and which are sensitive to endonuclease III, do not contribute significantly to the damage. This is consistent with earlier observations that singlet oxygen modifies predominantly guanine residues (42).

So far, FPG protein has been established as an enzyme which recognizes imidazole ring-opened purines (formamidopyrimidines). These modifications are generated from N^7-alkyl-purines in DNA by treatment with alkali and are on the same oxidation level as the original purines. Formamidopyrimidines are also generated in high yields in consequence of a reaction of hydroxyl radicals with DNA (6), probably by reduction of a radical formed by ·OH addition to C8 of the purine system (43).

The formation of formamidopyrimidines (Fapy) by singlet oxygen would be an indication that singlet oxygen gives rise to a similar one-electron oxidation-reduction sequence in the purine system. Superoxide generated by one-electron reduction of singlet oxygen could act as an intermediate in this reaction:

$$\text{Purine} + {}^1O_2 \rightarrow \text{Purine}^{+\cdot} + O_2^{-\cdot}$$
$$\text{Purine}^{+\cdot} \rightarrow \text{Fapy}^{+\cdot}$$
$$\text{Fapy}^{+\cdot} + O_2^{-\cdot} \rightarrow \text{Fapy} + {}^3O_2$$

The proposed mechanism is in accordance with our earlier observation (28) that formation of DNA single strand breaks by methylene blue and light is greatly enhanced in the presence of Fe^{3+}-EDTA in a reaction that involves both singlet oxygen and H_2O_2 : Under these conditions, superoxide is trapped by Fe^{3+}-EDTA, and the DNA damage is mediated by hydroxyl radicals generated in a Fenton reaction.

At the present time, it can not be excluded that modifications other than formamidopyrimidines are also recognized by FPG protein. In recent reports (44,45), the generation of 8-hydroxyguanosine residues in DNA by methylene blue in the presence of light has been described. The ratio between single strand break generation and 8-hydroxyguanosine formation observed in these studies is similar to the ratio observed here between single strand breaks and FPG protein-sensitive sites. Therefore, the possibility exists that FPG protein also recognizes 8-hydroxyguanosine. Neither can it be excluded that products of transient 4,5- or 4,8-endoperoxides of guanine, e.g. cyanuric acid derivatives, which have been detected after exposure of a guanosine derivative to singlet oxygen and which are presumably characteristic for singlet oxygen (27), are recognized by FPG protein.

Proteins similar to FPG have been shown to be present in other species, e.g. *Salmonella typhimurium, Micrococcus luteus* and *Bacillus subtilis* (46). Our results support the assumption that the biological role of FPG protein and its analogues is repair of oxidative DNA damage. By recognizing modified purine residues it might complement the redoxyendonucleases (endonunuclease

III in *E. coli*) which are restricted in their substrates to modified pyrimidines.

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