Accelerated Publications

The Other Function of DNA Photolyase: Stimulation of Excision Repair of Chemical Damage to DNA†

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ABSTRACT: DNA photolyase is a light-dependent DNA repair enzyme. It binds to cyclobutane pyrimidine dimers (Pyr⟨⟩Pyr) in DNA and upon excitation with a blue light photon splits the cyclobutane ring and restores the pyrimidines to native forms. The enzyme is specific for pyrimidine dimers, and it is not known to catalyze any other reaction either in ground or in excited state. However, when photolyase binds to Pyr⟨⟩Pyr but cannot catalyze repair because of lack of photoreactivating light, it still aids DNA repair by stimulating the nucleotide excision repair system. Recently, it was found that yeast photolyase binds to other lesions in DNA. In particular, the binding to cisplatin damaged DNA was highly specific. However, *in vivo* experiments revealed that this binding, in contrast to Pyr⟨⟩Pyr binding, did not stimulate but actually inhibited the removal of cisplatin damage by excision repair and hence photolyase sensitized cells to killing by cisplatin. In the present study, it is demonstrated that *Escherichia coli* DNA photolyase binds specifically to cisplatin 1,2-d(GpG) intrastrand cross-link and stimulates the removal of the lesion by *E. coli* excision nuclease *in vitro*. In agreement with the in vitro data, *in vivo* experiments revealed that photolyase makes cells more resistant to cisplatin killing.

Photolyase is widely distributed in nature but is absent in many species in an unpredictable manner (Harm, 1976). In particular, the enzyme is found in the enteric bacterium *Escherichia coli* which is not exposed to Pyr⟨⟩Pyr producing UV in its natural habitat (although it might be exposed in transit) but is absent in the soil bacterium *Bacillus subtilis* which receives extensive damage from sunlight. Similarly, humans and other placental mammals lack the enzyme (Cook & McGrath, 1967; Cleaver, 1968; Li *et al.*, 1993), but the enzyme is found in most other animals including marsupial mammals (Cook, 1970). Finally, in animals which do express photolyase no obvious correlation has been found between the level of the enzyme in various tissues and the potential for formation of Pyr⟨⟩Pyr in these tissues. Of special note, all of the internal organs contain photolyase, and it has been reported that brain has the highest photolyase activity of all tissues tested in chicken and opossum (Cook, 1970). The unpredictable distribution of the enzyme among species and among the different tissues of a given species have led to speculations that photolyase may in fact carry out a second function more consistent with its distribution (Harm, 1976).

An important clue for a nonphotoreactivation function of photolyase came from the observation that the enzyme, in the absence of photoreactivating light, bound Pyr⟨⟩Pyr and stimulated the removal of UV damage *in vivo* (Yamamoto *et al.*, 1983; Sancar & Smith, 1989) and *in vitro* (Sancar & Smith, 1989; Sancar *et al.*, 1984) and hence was capable of contributing to cellular defense against DNA damage even in the absence of light. However, these observations did not provide an answer to the question of what photolyase might do in organisms or tissues where the possibility of pyrimidine dimer formation is essentially nonexistent.

A recent report provided another clue for what the photoreactivating enzyme might do in the dark. It was found that yeast photolyase, in addition to UV-irradiated DNA, bound DNA damaged by many other agents albeit with considerably lower affinity than UV-irradiated DNA (Fox *et al.*, 1994). However, DNA damaged by cisplatin was bound by photolyase particularly tightly; it was estimated that the affinity of photolyase for cisplatin adducted DNA was only about 50-fold lower than its affinity for Pyr⟨⟩Pyr (Fox *et al.*, 1994). This raised the possibility that photolyase, which is unable to reverse the cisplatin damage with or without light (see below) may contribute to cellular resistance to cisplatin damage by binding to cisplatin adducts and facilitating their removal by excision nuclease as has been shown to occur for Pyr⟨⟩Pyr in yeast (Sancar & Smith, 1989). However, *in vivo* experiments with cisplatin revealed the opposite effect. Cells expressing photolyase (Phr^+) were more sensitive to killing by cisplatin than Phr^- cells leading to the conclusion that photolyase bound to cisplatin lesions which it could not repair and this binding interfered with the binding of the yeast excision nuclease which can repair cisplatin damage (Fox *et al.*, 1994).

We wished to conduct similar experiments with the *E. coli* photolyase-excision nuclease system using purified enzymes and a defined substrate (Sancar *et al.*, 1984). We obtained an unexpected result: photolyase stimulated the repair of

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cisplatin damage by the *E. coli* excision repair enzyme, the (A)BC excinuclease. We decided to characterize this surprising effect in more detail.

MATERIALS AND METHODS

Photolyase was purified as described (Sancar *et al.*, 1984), and its concentration was determined by absorption using the known extinction coefficient of the enzyme at 580 nm (Sancar, 1994). The UvrA, UvrB, and UvrC subunits of (A)BC excinuclease (Thomas *et al.*, 1985) were purified as described previously, and concentrations were determined using the Bradford assay (Bio-Rad).

Substrates for the DNA binding and excision assays were obtained by ligating a ^{32}P-labeled 12-mer with two other oligomers in the presence of 45 nt long complementary oligomer as described (Huang *et al.*, 1994). The duplexes were purified on 12% nondenaturing polyacrylamide gels, electroeluted, precipitated with ethanol, and resuspended in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 1 mM EDTA. DNA concentration was determined by Cerenkov counting using a specific activity of 7000 Ci/mmol for [γ-^{32}P]ATP (ICN).

For the binding assays, the reaction mixture (25 μL) contained 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 100 μg/mL bovine serum albumin, and the indicated concentrations of DNA and enzyme. The mixture was incubated at 22 °C for 30 min, glycerol was added to a final concentration of 5%, and the samples were loaded onto 5% polyacrylamide gel and electrophoresed at a constant voltage of 110 V for 2 h. Following electrophoresis the free and bound DNA were quantified by scanning the autoradiographs using a Molecular Dynamics Computing Densitometer 300 instrument.

For the excision assays, the reaction mixtures (150 μL) contained 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 10 mM dithiothreitol, and 100 μg/mL bovine serum albumin plus 10 nM substrate and 50 nM photolyase where indicated. After incubation at 22 °C for 30 min, UvrA (10 nM), UvrB (80 nM), and UvrC (40 nM) were added, and incubation was continued at 37 °C. At the indicated times 25 μL aliquots were taken and the reaction was stopped by adding EDTA to 15 mM. The DNA was extracted with phenol/chloroform, precipitated with ethanol, and separated on 8% polyacrylamide sequencing gels. The autoradiograms were scanned by densitometer for quantitative analysis. Under the conditions used, UvrA is limiting as determined empirically by varying the UvrA concentration in the reaction mixture.

For the *in vivo* assay of resistance to cisplatin, cultures of *E. coli* strains UNC524 (*phr::kan*) and NM522 (*phr*$^+$) were grown in Luria Broth for 16 h. Cells were collected by centrifugation, washed with phosphate buffered saline, and diluted to $A_{600} = 0.7$. Cisplatin was added at the indicated concentrations, and the cells were incubated at 30 °C for 2 h. Serial dilutions were plated on Luria agar plates, and the colonies were counted after 24 h at 37 °C.

RESULTS

First, we tested the binding of a 45 bp duplex containing a single cisplatin−1,2-d(GpG) diadduct, the major DNA lesion produced by cisplatin (Lippard & Berg, 1995). Figure 1 shows that the enzyme binds to this lesion with a $K_D \sim 5 \times 10^{-8}$ M, which is within a factor of 10 of the binding constant for thymine dimer (Sancar, 1994a). To ascertain that this binding was due to the cisplatin lesion in DNA and not to Pyr⟨⟩Pyr accidentally introduced into the oligomers during substrate preparation and handling, we exposed the photolyase−DNA mixture to photoreactivating light before loading onto the gel. Under these conditions the Pyr⟨⟩Pyr containing DNA is repaired and is released from the enzyme. The photoreactivating light had no effect on the photolyase−(Pt-DNA) complex (data not shown), consistent with our conclusion that binding was caused by the cisplatin adduct and furthermore that photolyase cannot repair cisplatin−1,2-d(GpG) diadduct. Furthermore, Dnase I footprinting assay revealed a footprint centered around the cisplatin adduct (data not shown) providing further evidence that the specific binding was due to cisplatin.

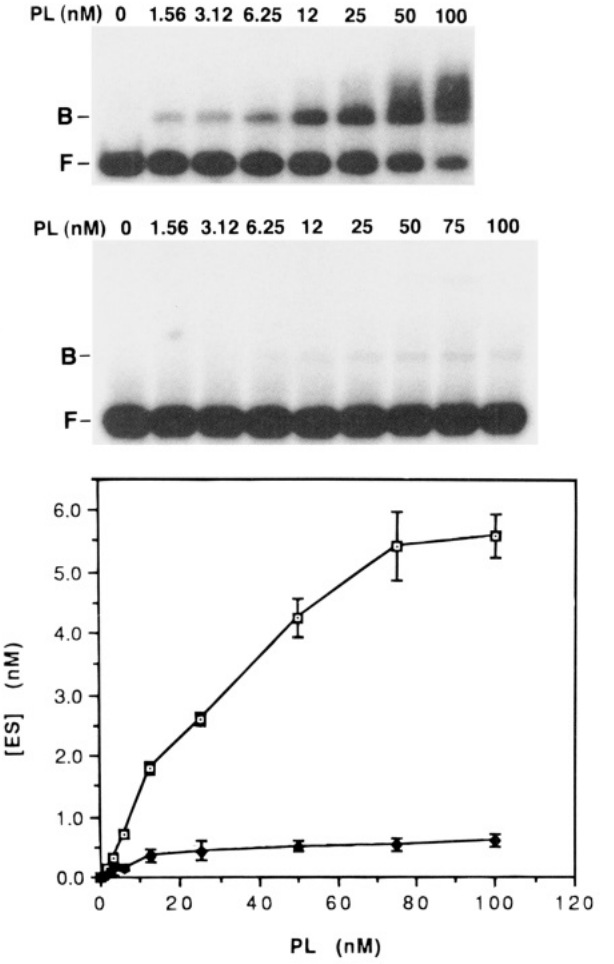

FIGURE 1: Binding of photolyase (PL) to 45 bp duplex with a cisplatin−1,2-d(GpG) adduct. (top) Gel retardation assay. Photolyase was incubated with 10 nM substrate for 30 min at 22 °C, and then the protein−DNA complex was separated on 5% nondenaturing polyacrylamide gel. F, free DNA; B, photolyase-bound DNA. (middle) Gel retardation assay with control DNA. The 45 bp duplex of identical sequence but no cisplatin adduct was mixed with photolyase and incubated as described. DNA complexes were separated under the same conditions as with the damaged DNA. (bottom) Binding isotherms. Data points from three experiments conducted under identical conditions as described in Materials and Methods were averaged. Open symbol, cisplatin-1,2-d(GpG) containing duplex; closed symbol, control 45 bp duplex with no adduct. The bars indicate standard error.

Having demonstrated specific binding of photolyase to the cisplatin−DNA adduct, we tested the effect of this binding on *E. coli* (A)BC excinuclease, which is known to excise

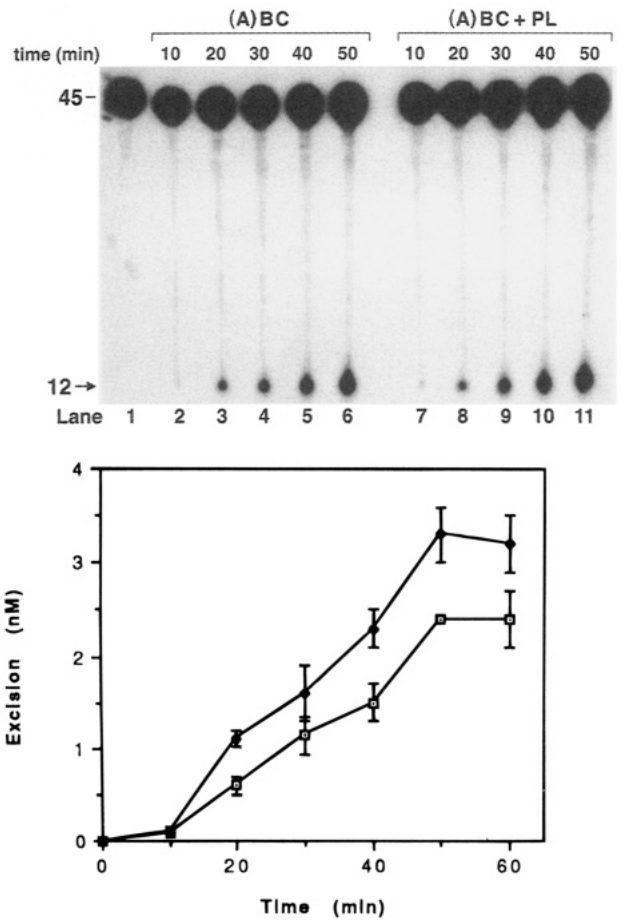

FIGURE 2: Photolyase stimulates the excision of cisplatin−1,2-d(GpG) adduct by (A)BC excinuclease. (top) Analysis of the reaction products by gel electrophoresis. (A)BC, mixture of UvrA, UvrB, and UvrC proteins; PL, *E. coli* photolyase. Lane 1 contains DNA alone. Samples in lanes 2−6 contain DNA that was incubated with UvrA, B, and C for the indicated times. Lanes 7−11, the DNA was incubated with photolyase for 30 min before adding UvrA, B, and C and incubating at 37 °C for the indicated time periods. The substrate was prepared as described in Materials and Methods, and it contains ^{32}P label at the sixth phosphate 5′ to the lesion. (A)BC excinuclease hydrolyzes the eighth phosphodiester bond 5′ and the fourth phosphodiester bond 3′ to the cisplatin adduct and releases 12 nt long radiolabeled fragment with the lesion. (bottom) Quantitative analysis of the excision reaction. Data from the experiment shown in panel a and two additional experiments conducted under identical conditions were averaged. Open square, excision with (A)BC excinuclease alone; closed diamond, excision in the presence of photolyase. The bars indicate standard error.

cisplatin adducts from DNA (Beck *et al.*, 1985; Visse *et al.*, 1991). The results show that, contrary to our expectations based on the yeast photolyase/excision nuclease system, *E. coli* photolyase in fact stimulates (A)BC excinuclease (Figure 2). The stimulation of excision of cisplatin (about 1.3-fold increase in rate) is smaller than that observed with Pyr⟨⟩Pyr(3-fold), which may explain the failure to observe stimulation in a previous study which used a less sensitive assay (Sancar *et al.*, 1984). Nevertheless, the stimulation observed with the excision assay used in this study was statistically significant, was quite reproducible, and was observed under a variety of conditions and two different cisplatin substrates, leading us to conclude that the observed effect was not an artifact of the particular set of conditions used and hence may be of relevance under physiological conditions.

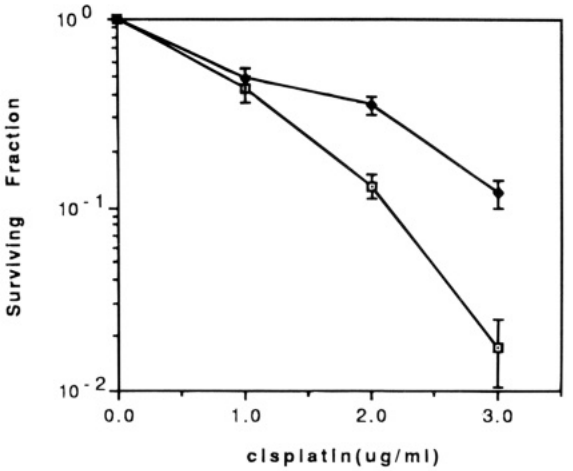

FIGURE 3: Photolyase increases the resistance of *E. coli* to cisplatin. Cultures of *E. coli* UNC524 (*phr::kan*, open symbol) and NM522 (*phr*$^+$, closed symbol) were grown in Luria Broth for 16 h. Cells were collected by centrifugation, washed with phosphate-buffered saline, and diluted to $A_{600} = 0.7$. Cisplatin was added at the indicated concentrations, and the cells were incubated at 30 °C for 2 h. Serial dilutions were plated on Luria agar plates, and the colonies were counted after 24 h at 37 °C. The data points are averages of three experiments, and the standard error is indicated.

To find out whether photolyase contributes to cellular resistance to cisplatin by stimulating excision repair, isogenic strains of *E. coli* differing only in the Phr phenotype were treated with increasing concentrations of cisplatin and the surviving fraction was measured. The results (Figure 3) clearly show that at all doses tested Phr$^+$ cells have higher survival than Phr$^-$ cells and that photolyase in *E. coli*, in apparent contrast with yeast, does not sensitize cells to cisplatin but actually contributes to cellular resistance to killing by this drug.

DISCUSSION

We do not have a satisfactory explanation for the different effects of photolyase on excision repair of cisplatin damage in *E. coli* and in yeast. It is significant, however, that the procaryotic and eucaryotic excision repair systems, aside from the dual excision mechanism, have very little in common, and the number and sequences of the subunits involved in excision are entirely different in the two systems (Prakash *et al.*, 1993; Sancar, 1994b; Friedberg *et al.*, 1995). Regardless of the cause of difference between yeast and *E. coli* with respect to photolyase−excision nuclease interplay, clearly in *E. coli* photolyase aids the bacterium in survival following cisplatin damage. A more extensive survey is needed to determine what other lesions are targets for photolyase-mediated facilitated excision repair.

Our results show that photolyase can participate in the repair of non-UV damage and in the absence of light. These findings may be relevant to why chicken and opossum brain (where neither the Pyr⟨⟩Pyr producing UV nor the blue light necessary for catalysis by photolyase can penetrate) contain high levels of photolyase. Further work with the photolyases and excision nucleases of these organisms will be necessary to test these predictions.

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