Molecular mechanisms of DNA repair inhibition by caffeine

[photolyase/(A)BC excinuclease/intercalating drugs]

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ABSTRACT   Caffeine potentiates the mutagenic and lethal effects of genotoxic agents. It is thought that this is due, at least in some organisms, to inhibition of DNA repair. However, direct evidence for inhibition of repair enzymes has been lacking. Using purified *Escherichia coli* DNA photolyase and (A)BC excinuclease, we show that the drug inhibits photoreactivation and nucleotide excision repair by two different mechanisms. Caffeine inhibits photoreactivation by interfering with the specific binding of photolyase to damaged DNA, and it inhibits nucleotide excision repair by promoting nonspecific binding of the damage-recognition subunit, UvrA, of (A)BC excinuclease. A number of other intercalators, including acriflavin and ethidium bromide, appear to inhibit the excinuclease by a similar mechanism—that is, by trapping the UvrA subunit in nonproductive complexes on undamaged DNA.

Caffeine has a myriad of pharmacological effects. Among these, the sensitization of cells to the lethal and mutagenic effects of DNA-damaging agents has been the subject of numerous studies in the last three decades (1–5). It has been suggested that caffeine binds to DNA—perhaps with higher affinity to damaged regions (6)—and thus interferes with the specific binding of repair enzymes (4). However, none of the repair proteins that have been purified and tested, *Micrococcus luteus* UV endonuclease (7), *Escherichia coli* 3-methyladenine DNA glycosylase I (8), and human placental AP endonuclease (9), were inhibited by caffeine. In *E. coli*, caffeine at 10–100 mM inhibits photoreactivation *in vivo* (3) and nucleotide excision repair *in vivo* (1) and in a permeabilized cell system (10).

Photoreactivation is the reversal of the mutagenic and lethal effects of far UV by subsequent exposure of cells to near UV or visible light (11). The phenomenon is mediated by photoreactivating enzyme, DNA photolyase. This enzyme repairs DNA by breaking the cyclobutane ring of pyrimidine dimers (80–90% of the total UV photoproducts) in a light-driven reaction. The enzyme binds to pyrimidine dimers in a light-independent reaction and, upon absorbing a photon of photoreactivating light (300–500 nm), donates an electron to the dimer, initiating an electronic reorganization which eventually produces two intact pyrimidines (11). In contrast to photoreactivation, which repairs pyrimidine dimers *in situ*, nucleotide excision repair entails the removal of a segment of the DNA backbone containing the damaged base(s) followed by filling in of the gap by DNA polymerase and sealing by ligase. In addition to removing pyrimidine dimers, this repair mechanism is responsible for removal of DNA adducts of a wide variety of chemicals such as psoralen, cisplatin, and mitomycin C (11). In *E. coli*, nucleotide excision is initiated by an ATP-dependent nuclease, the (A)BC excinuclease,* which incises the eighth phosphodiester bond 5' and the fourth phosphodiester bond 3' to the adducted nucleotide(s). In this study we have used defined DNA substrates, purified

photoreactivating enzyme, DNA photolyase, and the nucleotide excision repair enzyme (A)BC excinuclease to investigate the mechanism of repair inhibition by caffeine in *E. coli*. A preliminary account of this study has been published (12).

MATERIALS AND METHODS

Enzymes and Substrates. Photolyase and the three subunits of (A)BC excinuclease were purified as described previously (13, 14). The substrate for photolyase was a 48-base-pair (bp) duplex containing a centrally located thymine dimer and a ^{32}P label on the 5' terminus of the damaged strand (15). The substrate for (A)BC excinuclease was either UV-irradiated (130 J/m^2) ^3H-labeled pBR322 or a 137-bp duplex which was terminally labeled and contained a centrally located psoralen monoadduct (16).

DNase I "Footprinting." For photolyase footprinting, reaction mixtures (50 μl) containing 50 mM Tris·HCl at pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, bovine serum albumin at 100 μg/ml, 10 nM photolyase, ≈1 nM DNA substrate, and the indicated amounts of caffeine were incubated for 20–25 min in the dark. After incubation certain samples were exposed to photoreactivating light from a camera flash (17), MgCl$_2$ (10 mM) and CaCl$_2$ (5 mM) were then added to each sample, and the DNA was digested with DNase I (Bethesda Research Laboratories) at 12 ng/ml for 6 min at 23°C. The DNA was precipitated with ethanol and analyzed on 11% polyacrylamide sequencing gels.

The UvrA footprinting was carried out in 100 μl of ABC reaction buffer (50 mM Tris·HCl, pH 7.5/50 mM KCl/10 mM MgCl$_2$/2 mM ATP/1 mM dithiothreitol/50 μg of bovine serum albumin per ml) containing 6 nM UvrA, ≈2 nM DNA, and the indicated amounts of caffeine. The mixtures were incubated for 25 min at 23°C, then CaCl$_2$ (2.5 mM) and DNase I (5.6 ng/ml) were added, and after 5 min of incubation the DNA was precipitated with ethanol and analyzed on 8% polyacrylamide sequencing gels.

Incision Assay. This assay measures the conversion of supercoiled DNA to open circular form as a result of nicking by (A)BC excinuclease. The reaction mixtures (in 100 μl of ABC buffer) contained 170 fmol of UV-irradiated pBR322 (or nonirradiated control DNA), 170 fmol of UvrA, 6 pmol of UvrB, 12 pmol of UvrC, and, when indicated, 10 mM caffeine. The mixtures were incubated at 37°C and at time intervals 12.5-μl aliquots were taken and the reaction was stopped by adding SDS to 0.2%. The samples were heated at 65°C for 5 min and then the DNA was separated on 0.8% agarose gels, superhelical and open circular forms were located by staining with ethidium bromide, and the DNA was

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*We use the name "(A)BC excinuclease" for the nuclease activity resulting from the mixture of UvrA, UvrB, and UvrC proteins of *E. coli*. The UvrA subunit delivers UvrB to the damage site and then dissociates from DNA; the actual nuclease complex, therefore, includes only UvrB and UvrC; hence A is in parentheses to indicate that it is essential for the nuclease activity but is not present in the actual nuclease complex.

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cut out and quantified by scintillation counting. The average number of incisions per plasmid was calculated by using the Poisson distribution (18).

RESULTS AND DISCUSSION

Inhibition of Photoreactivation. The effect of caffeine on photolyase was investigated by DNase I footprinting on a 48-bp-long duplex containing a uniquely located thymine dimer. The result is shown in Fig. 1. In the absence of caffeine photolyase binds to the thymine dimer, producing a DNase I footprint of about 15 bp (lane 2). When the photolyase/DNA mixture is exposed to an intense flash of photoreactivating light, nearly all of the substrate is repaired and treatment with DNase I (i) reveals two new bands that are diagnostic of repaired substrate (the lower of these two bands is marked with the letter A) and (ii) fails to show any footprint as evidenced by the reappearance of band B, which was nearly completely absent in the nonphotoreactivated lane. Band C in Fig. 1 is unaffected by photolyase and is used as an internal
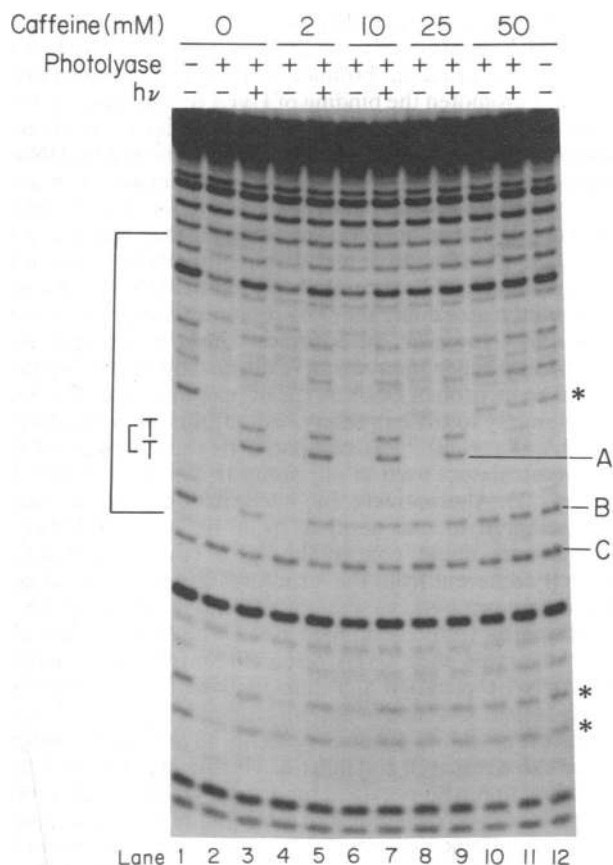

FIG. 1. Caffeine inhibits the specific binding of photolyase to DNA. Photolyase (10 nM) was mixed with substrate (≈1 nM) in 50 μl of reaction buffer containing the indicated amounts of caffeine. After incubation for 25 min in the dark certain samples were exposed to a flash of photoreactivating light (hν), then all DNA samples were digested with DNase I and analyzed on an 11% polyacrylamide sequencing gel. T̂T, thymine dimer; band A is generated by hydrolysis of the phosphodiester bond which joins the thymines that make up the dimer and its intensity is a measure of photorepair; band B is protected against DNase I digestion by photolyase and its extent of disappearance is a measure of photolyase binding; band C is generated by cleavage of a phosphodiester bond outside the photolyase binding site and it may be used as an internal control in comparing the intensities of band A and band B between different lanes. Caffeine slightly inhibited DNase I digestion at sites indicated by asterisks. These results were reproduced in several independent experiments.

standard in comparing lanes to one another. In the presence of caffeine the photolyase footprint gradually disappears with increasing concentrations of the drug (compare the intensities of band B in lanes 2, 4, 6, 8, and 10) and there is a corresponding decrease in photorepair as evidenced by decreasing intensity of band A in lanes 5, 7, 9, and 11 as compared with lane 3. Densitometric scanning of bands A and B (using band C as an internal standard) indicate that the decrease in intensity of band A (repair) is associated with an increase in band B (binding). Thus it appears that caffeine inhibits photorepair by interfering with photolyase binding but has no effect on the photochemical reaction per se.

Inhibition of (A)BC Excinuclease. Damage recognition and removal by (A)BC excinuclease occurs in several stages (18): the damage-recognition subunit, UvrA, binds to DNA containing pyrimidine dimers or other lesions, delivers the UvrB subunit to the damaged site, and dissociates from DNA; the UvrC subunit then binds to the UvrB/damaged DNA complex and incisions are made on both sides of the DNA adduct and at a distance from it. Caffeine inhibits nicking of damaged DNA by the enzyme both in permeabilized cells (10) and in vitro (Fig. 2).

Since several steps in the reaction mechanism are ATP dependent we considered the possibility that the purine analog caffeine may inhibit the enzyme as a competitive inhibitor of ATPase function. However, we found (G. Myles and A.S., unpublished data) that 10 mM caffeine has no effect on the ATPase activity of UvrA, in agreement with permeabilized cell experiments which indicated that the inhibition of nicking was not reversed with higher ATP concentrations (10). Therefore, we considered alternative mechanisms of inhibition such as interference with binding of UvrA, the loading of UvrB, the binding of UvrC to the UvrB/DNA complex, or incision. To identify the particular step inhibited by the drug we added caffeine to incision reaction mixtures
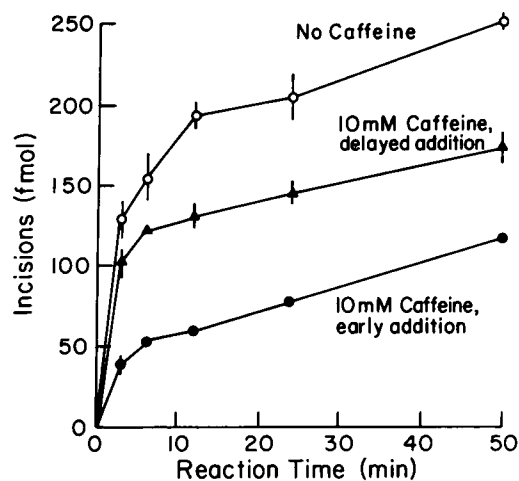

FIG. 2. Caffeine inhibits incision of UV-irradiated DNA by (A)BC excinuclease. The mixtures (100 μl) were incubated at 37°C and at the indicated times 12.5-μl samples were withdrawn to quantify incision. ○, No caffeine was added; DNA was incubated with UvrA and UvrB for 12 min and then the incision reaction was initiated ("zero time") by the addition of UvrC. ●, Caffeine was incubated with DNA for 12 min, UvrA plus UvrB was added, and after further incubation for 12 min UvrC was added. ▲, DNA was incubated with UvrA and UvrB for 12 min and then caffeine and UvrC were added. The means and standard errors plotted are from two experiments carried out under indentical conditions. The background incisions (with undamaged DNA), which ranged from 10 (no caffeine), 0 (early addition), and 7 fmol (delayed addition) at 3 min to 52 (no caffeine), 28 (early addition), and 14 fmol (delayed addition) at 50 min, have been subtracted from the specific incisions plotted in this figure. Note that these values demonstrate that caffeine does not promote incision of undamaged DNA.

at various stages of assembly of the enzyme. As is seen in Fig. 2, maximum inhibition is achieved when the drug is added prior to loading of UvrB onto DNA, which implies that caffeine interferes with binding of UvrA, delivery of UvrB to the damaged site, or both. Some inhibition is observed even with delayed addition of caffeine which might be interpreted as interference with the binding of UvrC or incision. While our experiment does not categorically preclude such an explanation, the most likely cause of the low inhibition with delayed addition of caffeine is that the delivery of UvrB to DNA is actually incomplete in 12 min and it continues at a slower rate for longer times (D. K. Orren and A.S., unpublished observation). Therefore, addition of caffeine 12 min after UvrA and UvrB would still result in some interference with loading of UvrB and therefore incision.

Effect of Caffeine on UvrA Binding. We investigated the binding of UvrA to DNA by DNase I footprinting, using a 137-bp-long duplex containing a uniquely located psoralen monoadduct (16, 19). The results are shown in Fig. 3. In the absence of caffeine UvrA binds specifically to the substrate, yielding a footprint of 33 bp (lane 10); no nonspecific binding is seen at the UvrA concentration (6 nM) used in these experiments. Caffeine does not appear to interfere with the formation of the damage-specific complexes, as indicated by the presence of a discernible footprint at concentrations of up to 5 mM (lane 14). Instead, it strongly promotes nonspecific binding by UvrA as evidenced by the striking protection of the whole fragment (damaged or undamaged) from DNase I digestion when both UvrA and caffeine are present. We can also detect caffeine-dependent binding of UvrA to undam-
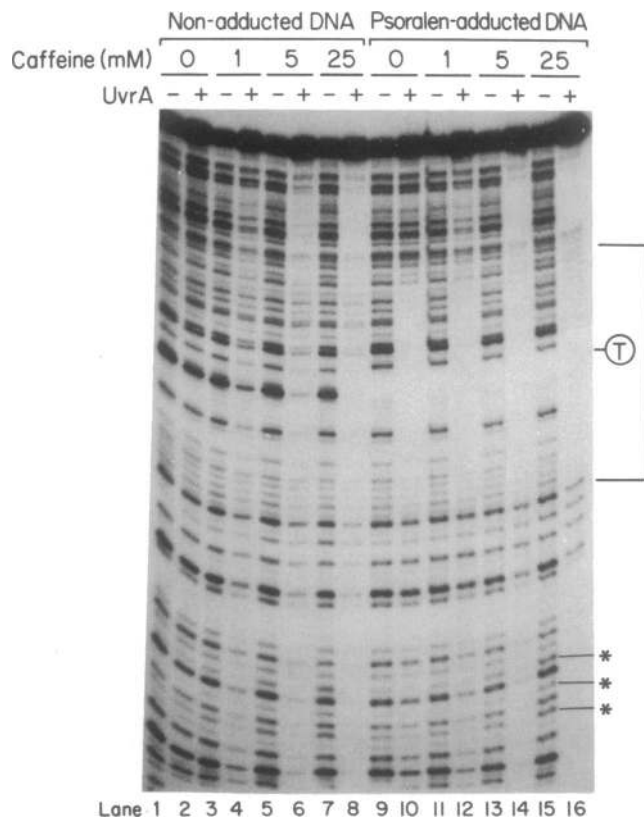

FIG. 3. Caffeine promotes the binding of UvrA to undamaged DNA. The 137-bp synthetic duplex with or without a psoralen monoadduct was mixed with UvrA in the presence of 0–25 mM caffeine, digested with DNase I, and analyzed on an 8% polyacrylamide sequencing gel. The bracket indicates the specific UvrA footprint (lane 10) and the circled T the location of psoralen-adducted thymine (75 nucleotides from the labeled 5′ terminus). Caffeine slightly enhanced DNase I digestion at sites indicated by asterisks.

aged DNA by using the gel-shift assay (data not shown). Binding at these sites is nonproductive; that is, it does not lead to incision upon addition of UvrB and UvrC subunits as is apparent from the control data listed in the legend of Fig. 2. Furthermore, the presence of UvrB in the reaction mixture did not alter UvrA's high-affinity binding to undamaged DNA (data not shown). Thus we conclude that caffeine inhibits (A)BC excinuclease by creating additional binding sites for UvrA which compete with the repairable DNA damage. An additional effect of caffeine on the delivery of UvrB to damaged sites, though unlikely, cannot be excluded by our experiments.

Effect of Other Intercalators on UvrA Binding. In considering how caffeine traps UvrA we noted that the drug reportedly intercalates into nucleic acids (20–22), and it appears to affect the DNase I digestion pattern in our experiments in the same way that other intercalating compounds affect DNase I digestion: producing a sequence specific enhancement or inhibition of digestion (23, 24). These caffeine-enhanced or inhibited digestion sites are indicated by asterisks in Figs. 1 and 3. Therefore, we tested other intercalators, including some which are known to inhibit DNA repair in vivo (1, 25), for their effect on UvrA. We found that ethidium bromide at 1.0 μM and chloroquine at 0.2 mM promoted the binding of UvrA to undamaged DNA to the same level as 25 mM caffeine under experimental conditions similar to those in Fig. 3 and as tested by DNase I footprinting. Comparable results were obtained with acridine orange and acriflavine by using the gel-shift assay (data not shown). These observations suggest that caffeine promotes UvrA binding by intercalating into DNA. In line with this conclusion, it has recently been reported that the bifunctional intercalator ditercalinium promotes binding of UvrA to DNA (26). However, in contrast with our findings with caffeine and other intercalators, ditercalinium did promote enzymatic incision of DNA by (A)BC excinuclease. This may be due simply to the extremely high affinity of ditercalinium to DNA ($K_d = 10^{-7}$ M) compared to the affinities of the monointercalators used in our study ($K_d \approx 10^{-3}$ to 10^{-5} M) (see ref. 27). Alternatively, the intercalation of two aromatic rings tethered to one another by a 10- to 15-Å linker in ditercalinium could conceivably give rise to a structure distinctly different from the structures of monointercalators and thus contribute to the differential handling of these structures by (A)BC excinuclease. In addition, it has also been suggested that UvrA/ditercalinium/DNA complexes block DNA replication and thus contribute to toxicity (26).

Conclusion. Caffeine is perhaps the most extensively consumed drug, and its genotoxic effects have been the subject of extensive research and debate. The results presented here in conjunction with previous reports on DNA–caffeine interactions lead us to conclude that many of the pleiotropic genetic effects of caffeine may be ascribed to the intercalation of the drug into DNA, which in E. coli causes inhibition of photoreactivation by blocking specific enzyme binding and inhibition of excision repair by increasing nonspecific enzyme binding. We have also shown that caffeine at 10 mM inhibits nucleotide excision repair in a HeLa cell-free extract system (12). However, a 60-kg person must consume 75–100 cups of coffee within a short period to achieve 10 mM caffeine in the blood. The caffeine in this amount of coffee is about 10 g, which is fatal in humans when taken all at once (28). Therefore, whether the DNA-repair-inhibiting activity of caffeine is significant for its pharmacological and toxicological effects cannot be ascertained from the available data.

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