Perturbations of Enzymic Uracil Excision due to Guanine Modifications in DNA

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ABSTRACT

Phage PBS2 DNA, which contains uracil in place of thymine, was used as substrate for purified Bacillus subtilis uracil:DNA glycosylase. Incubation of this DNA with the ultimate carcinogen N-acetoxy-N-2-acetylaminofluorene resulted in the production of N-(deoxyguanosin-8-yl)acetylaminofluorene. A decreased $V_{\text{max}}$ resulted from the reaction of the glycosylase with this arylamidated substrate. Addition of a 2-fold excess of control PBS2 DNA following initiation of the reaction with the modified substrate showed delayed dissociation of the enzyme from the arylamidated DNA. This shows that the presence of a carcinogen-modified DNA base can reduce the capacity for uracil excision. Therefore, interference with enzymic release of uracil from DNA may be an indirect mechanism of mutagenesis by carcinogen:DNA adducts.

INTRODUCTION

The presence of uracil in DNA may result from incorporation in place of thymine during DNA synthesis or from hydrolytic deamination of DNA cytosines. Such deaminated cytosines, if left unrepaired, result in transition mutations (6, 7). Such uracils are removed from DNA by the enzyme dUra(DNA)glycosylase, which has been found in a wide variety of species (14). Since deamination of cytosine can occur under physiological conditions (16), dUra(DNA)glycosylase activity is necessary to preserve genomic integrity in the face of continuous mutagenic damage. Alterations of this activity can therefore result in mutagenesis.

The activities of various dUra(DNA)glycosylases towards their substrates are altered by the presence of other types of DNA damages. These include uracil dimers (4, 13), 5-fluorouracils (2, 12, 23), apurinic sites (5), apyrimidinic sites (22), and 8-2-hydroxy-2-propylpurines (5). The latter form of damage, although not detected in vivo, resembles the predominant form of DNA alteration caused by the carcinogen AAAF (17). This raises the possibility that DNA adducts of this carcinogen might effect enzymic excision of DNA uracils. Therefore, the effects of AAAF-modified guanines in DNA on dUra(DNA)glycosylase were investigated. It was found that the enzymic $V_{\text{max}}$ was reduced if the substrate contained dGuo-AAF. DNA challenge experiments indicated that the basis for this effect is delayed release of the modified enzyme from the modified DNA. Therefore, enzymic uracil excision is reduced by the presence of covalent DNA adducts of a known carcinogen.

MATERIALS AND METHODS

DNA Preparation and Modification. Phage PBS2 was grown in Bacillus subtilis, and the DNA was purified and stored at 100 µg/ml according to the method of Lindahl et al. (15). PBS2 DNA was radiolabeled by addition of $^{3}H$-uridine, $^{14}C$-uridine, or $^{14}C$-guanosine as described previously (5). The DNA was reacted with AAAF (obtained from the National Cancer Institute Carcinogen Repository, Bethesda, MD) at 0.22 mg/ml for 40 min at 37°C according to the method of Yamasaki et al. (24). After extraction of the carcinogen with ether, the residual ether was evaporated by a stream of nitrogen, and the DNA was dialyzed into 10 mM Tris-HCl (pH 8.0):1 mM EDTA.

Analysis of DNA Damage. The degree of guanine modification in PBS2 DNA was assessed by quantitating the proportion of dGuo-AAF in $^{14}C$-guanosine-labeled DNA. After denaturation and enzymic digestion to deoxynucleosides by S1 nuclease and acid phosphatase (21), the hydrolysate was applied to a 4-ml Sephadex LH-20 column. Analysis of modified deoxyguanosines was then performed by column elution and subsequent thin-layer chromatography according to the method of Amacher et al. (1). Marker dGuo-AAF was synthesized by the method of Poirier (21).

Enzymology. dUra(DNA)glycosylase was purified from B. subtilis strain SB168 (purchased as a frozen paste from Grain Processing Corp., Muscatine, IA) according to the method of Cone et al. (3). The phosphocellulose was prepared according to the method of Greene et al. (6). The enzyme assays, including reaction conditions, identification of the released product, and analysis of the Lineweaver-Burk plot, were performed as described previously (4, 5). DNA challenge experiments were performed using both modified and unmodified PBS2 DNAs. Purified dUra(DNA)glycosylase (0.5 µl; diluted 1:10) was incubated with 0.75 nmol as nucleotide of $^{3}H$-uridine-labeled PBS2 DNA containing 2.6% dGuo-AAF in 75 µl of 10 mM Tris-HCl (pH 8.0):1 mM EDTA:10 µl of 100 mM KH$_2$PO$_4$ (pH 7.0):1 mM EDTA. After 5 min at 37°C, 1.5 nmol of unmodified $^{14}C$-labeled PBS2 DNA were added to a final volume of 100 µl, and uracil release was measured at various intervals as described previously (5).

RESULTS

The activity of B. subtilis dUra(DNA)glycosylase against PBS2 DNA reacted with AAAF is shown in Chart 1. The damaged DNA contained 2.6% of the guanines as dGuo-AAF. This decreased the enzymic $V_{\text{max}}$ by 33% as compared with the control undamaged PBS2 DNA. As with other types of damaged purines (5), no modified purines were released from DNA by the enzyme. There was no significant alteration of the $K_m$ when the enzyme was reacted with the arylamidated substrate.

The basis for this observed reduction in the $V_{\text{max}}$ was investigated by letting enzymic uracil release commence on the AAAF-modified DNA and then adding twice the amount of unmodified substrate. The results are shown in Chart 2. The rate of uracil release from the modified DNA is substantially reduced. Addition...
of control DNA in 2-fold excess did not initially affect the rate of uracil release from the modified substrate, and it was 30 min before significant activity against the second substrate was noted. This is in contrast to similar DNA challenges with unmodified DNA, where significant activity against an even smaller amount of the second substrate was evident 6 min after its addition (5). This demonstrates that the inhibition of dUra(DNA)glycosylase by dGuo-AAF in DNA is not irreversible but rather due to delayed dissociation of the enzyme from its substrate.

**DISCUSSION**

Previous experiments have shown dUra(DNA)glycosylase activity to be reduced by the presence of 8-(2-hydroxy-2-propyl)purines, predominantly guanines (5). These are introduced by photoalkylation, i.e., near-UV irradiation of DNA in the presence of isopropanol and a free radical photoinitiator (5, 17). The major product of the AAAF reaction with DNA is also a guanine modified at the C-8 position (9), and a similar effect was obtained here using such modified PBS2 DNA as substrate. This is the first reported DNA adduct of a known carcinogen that reduces enzymic uracil release. The 33% reduction of the dUra(DNA)glycosylase $V_{\text{max}}$ by 2.6% arylamidated guanines is notably less than the 75% reduction caused by 1.6% photoalkylated purines (5). It is possible that the isopropyl adduct might bind the glycosylase more efficiently than dGuo-AAF. Alternatively, the basis for this difference may lie in the greater heterogeneity of damage resulting from DNA photoalkylation (5). Conformations of the modified guanines may differ between the AAAF-reacted and the photoalkylated bases. The presence of dGuo-AAF in DNA results in rotation around the glycosylic bond, causing a shift from the anti to the syn conformation (9). While a similar change has been suggested to occur in photoalkylated DNA (17), the conformational studies necessary to establish this have not yet been performed.

The activity of rat brain DNA cytosine 5-methyltransferase has been studied using substrate containing dGuo-AAF (20). The extent of the $V_{\text{max}}$ reduction obtained with the methyltransferase was similar to that observed here for dUra(DNA)glycosylase. The basis for that inhibition was shown by mixing experiments to be blockage of the movement of the processive methyltransferase along the DNA helix by the modified guanine adducts. Once blocked at a dGuo-AAF, the enzyme becomes irreversibly bound (20). Similar results have been reported for DNA polymerases assayed with AAAF-modified templates (18, 19). This is not the case for dUra(DNA)glycosylase, which eventually does dissociate from the modified substrate. The difference may be related to the more completely processive characters of the DNA polymerases and the methyltransferase. In contrast, the dUra(DNA)glycosylase releases about 5% of the DNA uracils before dissociation rather than effecting quantitative uracil excision (5). Other factors may also affect the extent of processivity of repair enzymes, as has been demonstrated with the pyrimidine-dimer DNA glycosylase purified from T4-infected Escherichia coli (10). Further investigations with different enzymes are necessary to establish if reduction of excision of damaged bases by repair glycosylases is a common effect of dGuo-AAF in DNA.

In order to be mutagenic, DNA uracil must arise via cytosine deamination. This preferentially occurs in single-stranded DNA; the deamination rate in native DNA is 0.3 to 0.5% of that of denatured DNA (14). Similarly, denatured DNA is more susceptible to reaction with AAAF at the C-8 position of guanines than native DNA (9). Therefore, specific regions susceptible to cytosine deamination may also suffer increased in vivo damage by that carcinogen. Sequence variation within the genome must also be taken into account, since dGuo-AAA production is far greater in the alternating copolymer poly(dG-dC)-poly(dG-dC) reacted with AAAF than with the homopolymer poly(dG)-poly(dC) (11). Therefore, random introduction of dGuo-AAF cannot be assumed, and there is ample theoretical potential for clustering of such arylamidated guanines within specific genomic loci. These could then block entry of dUra(DNA)glycosylase into a nucleotide tract containing uracil and thereby obstruct initiation of base excision repair. This raises the possibility that inhibition of dUra(DNA)glycosylase by carcinogen-modified purines is an indirect source of in vivo transition mutations.

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