Enzymatic excision from γ -irradiated polydeoxyribonucleotides of adenine residues whose imidazole rings have been ruptured

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ABSTRACT

The main forms of base damage in polydeoxyadenylic acid γ -irradiated under hypoxic conditions are due to saturation and fragmentation of the adenine imidazole ring. An irradiated polymer was annealed with an equimolar amount of poly (dT) to generate a double-stranded polydeoxyribonucleotide containing scattered damaged base residues. On incubation of the latter with partially purified cell extracts of *E. coli*, imidazole ring-opened adenine, i.e. 4,6-diamino-5-formamidopyrimidine, was released in free form by a DNA glycosylase activity. The enzyme has been purified 4,500-fold, has $M_r = 29,000$, and appears to be identical with the previously described DNA repair enzyme formamido-pyrimidine-DNA glycosylase.

INTRODUCTION

X-irradiation was the first environmental agent demonstrated to increase the frequency of mutation (1). It has since been shown to cause several types of lesions in DNA, including single- and double-strand breaks, base-free sites, and alterations of the nitrogenous bases (2,3). Though radiation-induced base damage in DNA is known to make an important contribution to the mutagenic and lethal effects observed in vivo (4), for technical reasons most studies on DNA lesions induced by ionizing radiation have until recently been concerned with the formation and fate of strand breaks. Ionizing radiation is thought to damage bases through generation of hydroxyl radicals, to which pyrimidine residues are somewhat more sensitive than purine residues (5). A DNA glycosylase which catalyzes the excision of ring-saturated, fragmented and ring-contracted thymine bases from oxidized and γ -irradiated DNA has been found both in bacteria and in

mammalian cells (6-9). However, this enzyme does not act on altered purine residues (10). It has been shown for adenine damaged by ionizing radiation that the two main products formed in DNA are 8-oxoadenine (saturation of the imidazole ring) and 4,6-diamino-5-formamidopyrimidine (fragmentation of the imidazole ring) (5,11).

Chetsanga and Lindahl (12) have described a DNA glycosylase which releases 7-methylguanine residues whose imidazole rings have been opened (2,6-diamino-4-hydroxy-5-N-methylformamido-pyrimidine) from alkylated DNA. This enzyme also occurs both in bacteria and mammalian cells (13), and the *E.coli* enzyme has been highly purified (14). While the enzyme has been termed formamido-pyrimidine-DNA glycosylase, it has not been known if this (or any other) DNA glycosylase could act on the ring-opened forms of unmethylated adenine and guanine generated by radiolysis (Fig. 1). In the present paper, the enzymatic excision of such γ -irradiation products of adenine residues is investigated.

MATERIALS AND METHODS

Synthesis of polydeoxyribonucleotides

Poly(dA) and poly(dT) were synthesized using calf terminal deoxynucleotidyl transferase (15) purchased from PL Biochemicals. [$8\text{-}^3\text{H}$] dATP ($24.3 \text{ Ci} \cdot \text{mmol}^{-1}$) and [$\text{U-}^{14}\text{C}$] dATP ($450 \text{ mCi} \cdot \text{mmol}^{-1}$) were obtained from Amersham. The poly (^3H]dA) had a specific activity of $700,000 \text{ cpm} \cdot \text{nmol}^{-1}$ and the poly (^{14}C]dA) $8,500 \text{ cpm} \cdot \text{nmol}^{-1}$.

Irradiation of poly(dA)

Radioactively labelled poly(dA) was dialysed against 1 mM potassium phosphate, pH 7.4, which had been degassed and then saturated with nitrogen. The polymer was diluted in this buffer to a final concentration of 0.2 mM. The solution was sealed in a microreaction V glass vial (Wheaton Scientific, Millville, N.J., USA) under an atmosphere of N_2 , and irradiated with a dose of 200 Gy at 8°C to 12°C using a ^{60}Co source of flux $37 \text{ Gy} \cdot \text{min}^{-1}$ (Gamma beam 650 - Atomic Energy of Canada Ltd., Ottawa, Canada). The polymer was precipitated with two volumes of ethanol, in the presence of 0.5 M NaCl,

Fig.1. Ring-opened forms of adenine and the alkylation product 7-methylguanidine in DNA. I: Adenine; II: 4,6-diamino-5-formamidopyrimidine; III: 7-methylguanidine; IV: 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine. Note that these formamidopyrimidines are not attached to deoxyribose in the same fashion as naturally occurring pyrimidines in DNA.

redissolved and then dialysed at 4°C against 1 M NaCl/10 mM potassium phosphate, pH 7.4 for 20 h, followed by 0.1 mM EDTA/10 mM Tris · HCl, pH 7.4, for 4 h with several changes. The recovery of radioactive polymeric material after irradiation and dialysis was 60%. Aliquots for base analysis of irradiated polymers were hydrolysed with formic acid at 60°C for 16 h (11).

Reference compounds

8-Oxoadenine was prepared according to the method of Ikehara *et al.* (16), and the imidazole ring opened form of 7-methylguanidine was made as described by Haines *et al.* (17). 4,6-Diamino-5-formamidopyrimidine was obtained from 4,5,6-triaminopyrimidine (Sigma) by reaction with formic acid at 56°C for 2 h, followed by removal of the formic acid through evaporation under reduced pressure (18). As expected, the formic acid treatment of triaminopyrimidine generated a compound with the HPLC fractionation properties of 4,6-diamino-5-formamido-pyrimidine (11). DNA containing ring opened 7-methylguanidine was prepared as described by Chetsanga and Lindahl (12).

Enzymatic release of radiolysis products of adenine

The irradiated poly(dA) was annealed with an equimolar amount of poly(dT) to generate a double-stranded polymer. The standard enzyme reaction mixture (50 μ l) contained 0.1 M KCl/0.07 M Hepes $\cdot$ KOH, pH 7.8/1 mM EDTA/1 mM dithiothreitol/1 nmol of double-stranded, 3 H-labelled polydeoxyribonucleotide/50ng (10^{-10} units, ref.12) of purified *E.coli* formamido-pyrimidine-DNA glycosylase. After 20 min at 37°C, the reaction mixtures were chilled to 0°C and 5 μ l 2 M NaCl, 10 μ l heat-denatured calf thymus DNA (20mg $\cdot$ ml $^{-1}$) and 150 μ l cold ethanol were added. After 20 min at -70°C, the samples were centrifuged for 15 min in an Eppendorf centrifuge and 150 μ l of each supernatant were recovered. These solutions were reduced to dryness under vacuum and redissolved in H $_2$ O for chromatographic analysis.

The enzymatic liberation of ring-opened 7-methylguanine residues from alkylated DNA was assayed as described (12).

Analytical chromatography

Reverse phase HPLC analysis was performed on a Varian 5000 Liquid Chromatograph equipped with a MCH-10 column (4 mm x 30 cm, Micro-Pac). The column was eluted with distilled H $_2$ O at 2 ml $\cdot$ min $^{-1}$ for the first 10 min, and then with a 0-100% linear methanol gradient at 1 ml $\cdot$ min $^{-1}$ for 20 min. Ion-exchange HPLC analysis was performed isocratically at 2 ml $\cdot$ min $^{-1}$ on a Partisil 10/25 SCX column (Whatman) with 25 mM ammonium phosphate (pH 3.5) as eluant. In either case 1 ml fractions were collected. Descending paper chromatography on Whatman 3MM paper (7) was with n-butanol: formic acid: water, 10:2:15. The solvent was allowed to run off the paper to increase separation. (R_f values were: Adenine 0.21, 4,6-diamino-5-formamidopyrimidine 0.07, and 8-oxoadenine 0.27).

Enzyme purification

Highly purified formamidopyrimidine-DNA glycosylase was obtained as a side fraction during the purification of endonuclease III (10). The enzyme activity was measured with an alkylated DNA substrate containing ring opened 7-methylguanine residues (12). Briefly, a crude cell extract of *E.coli* B was treated with Polymin-P and fractionated step-

wise by phosphocellulose chromatography as described previously (10). The 15-fold purified activity was then fractionated by linear gradient chromatography on a single-stranded DNA-cellulose column. The most active fractions appeared at 0.5 M NaCl, and they were pooled and concentrated by ultrafiltration. This 600-fold purified material was then further fractionated by AcA-54 gel filtration. The most active fraction after this step was approximately 4,500-fold purified, and was obtained in 10% yield.

RESULTS

4,6-Diamino-5-formamidopyrimidine residues are formed by γ -irradiation of poly(dA)

Radioactively labelled poly(dA) which had been γ -irradiated, ethanol precipitated and dialysed was hydrolysed with formic acid and analysed by HPLC. About 0.16% of the adenine was converted into 4,6-diamino-5-formamidopyrimidine by a dose of 200 Gy. When [U-¹⁴C] labelled, instead of [8-³H] labelled, poly(dA) was irradiated, no significant difference in the yield of 4,6-diamino-5-formamidopyrimidine was observed. The ring-opened form of adenine is a γ -irradiation product as it was not detected in hydrolysates of unirradiated, freshly prepared polymers. However, [8-³H] adenine labelled poly (dA) gradually accumulated base radiation products during storage, as noted previously by Bonicel *et al.* (11).

Enzymatic release of free 4,6-diamino-5-formamidopyrimidine from irradiated poly(dA)

When irradiated poly(dA), which had been made double-stranded by the addition of an equimolar amount of poly(dT), was incubated with highly purified formamidopyrimidine-DNA glycosylase, free 4,6-diamino-5-formamidopyrimidine was detected after ethanol precipitation and analysis of supernatant by reverse phase HPLC (Fig.2). The enzymatically released material also co-eluted with authentic 4,6-diamino-5-formamidopyrimidine on ion-exchange HPLC (Fig. 2) and co-migrated with this reference compound on paper chromatography (data not shown). Small amounts of material co-

Fig.2. Analytical chromatography of ethanol soluble material released from γ -irradiated poly(dA) by purified *E.coli* formamidopyrimidine-DNA glycosylase (50 ng protein added to the standard reaction mixture). The ^3H -labelled poly(dA) was exposed to 200 Gy under N_2 and then annealed with non-radioactive poly(dT) prior to incubation with the enzyme. (Top) Reverse phase HPLC, Micro-pac MCH-10 (C_{18}), (bottom) ion-exchange HPLC, Partisil 10/25 SCX. Brackets indicate the elution of authentic markers, a = 4,6-diamino-5-formamidopyrimidine, b = 8-oxoadenine, c = adenine and d = 2,4,6-triaminopyrimidine. Filled symbols, enzyme added; open symbols, no enzyme. The 4,6-diamino-5-formamidopyrimidine released enzymatically represents 0.03%, and the non-enzymatically liberated adenine 0.10%, of the total radioactivity in the reaction mixture. The two analyses were performed on different reaction mixtures.

Fig. 3. SDS-polyacrylamide gel electrophoresis of formamidopyrimidine-DNA glycosylase purified from *E.coli*. The protein samples were denatured by heating at 100°C for 90 s in the presence of 2% SDS and 5% mercaptoethanol, and then electrophoresed in Tris/glycine/SDS buffer, pH 8.3 (ref.22) on a 10% polyacrylamide slab gel together with reference proteins. The protein bands were visualised by staining with Coomassie blue. Lane a, chymotrypsinogen and ribonuclease; b, 4,500-fold purified enzyme; c, 600-fold purified enzyme. The enzyme fraction in lane b is estimated to be about 90% pure.

migrating with unmodified adenine were released non-enzymatically (Fig.2). This apparent release of adenine may be due to depurination, or represent reversion to adenine of a labile radiation product.

The release of 4,6-diamino-5-formamidopyrimidine is effected by the DNA glycosylase which liberates ring-opened 7-methyl-guanine.

The *E.coli* formamidopyrimidine-DNA glycosylase, an activity of about $M_r = 30,000$ (12, 14), has been purified 4,500-fold from *E.coli*. A single major protein band of $M_r = 29,000$ and 2-3 faint bands were observed on SDS-polyacrylamide gel electrophoresis (Fig. 3), indicating that the enzyme preparation was about 90% pure. This DNA glycosylase fraction catalyzed the release of both ring-opened 7-methyl-guanine and 4,6-diamino-5-formamidopyrimidine. The enzyme apparently liberated the former compound about 10 times more effectively than the latter one. However, these data may not be directly comparable as one substrate was derived from alkylated high molecular weight DNA, while the other was a

heavily irradiated synthetic polydeoxyribonucleotide. The ratio between the two activities remained unchanged when the 15-fold purified phosphocellulose fraction was compared with the 4,500-fold purified fraction obtained after additional chromatography by DNA-cellulose and gel filtration. (Assays in crude cell extracts were unreliable due to interfering activities). These data strongly indicate that the same enzyme was responsible for the excision of both kinds of ring-opened purine residues.

DISCUSSION

Ring-opened purines in DNA are of interest as potential cell-killing or mutagenic lesions after exposure to ionizing radiation. However, no direct evidence is available on their potential miscoding or non-coding properties. An apparently analogous lesion is generated in alkylated DNA as a secondary product of 7-methylguanine. Boiteux and Laval (19), using an alkylated polydeoxyribonucleotide containing the latter type of lesion, have shown that the ring-opened purine derivative blocks chain elongation by E.coli DNA polymerase I in vitro. Their data consequently indicate that the altered purine may be regarded as a cell-killing lesion.

The experiments described here have confirmed that one of the consequences of γ -irradiation is saturation and rupture of the imidazole ring of adenines in DNA. The extent of ring-opening observed as a function of radiation dose was similar to that observed by Bonicel et al. (11) for DNA, and by van Hemmen and Bleichrodt (20) for free adenine. In contrast, Chetsanga and Grigorian recently reported (21) a much greater extent of conversion, and they were unable to detect the enzymatic release of 4,6-diamino-5-formamidopyrimidine from γ -irradiated DNA using a partially purified preparation of E.coli formamidopyrimidine-DNA glycosylase. In the present work, the 4,6-diamino-5-formamidopyrimidine generated in a polydeoxyribonucleotide by γ -irradiation has been shown to be liberated by a DNA glycosylase activity. This appears to be a function of the previously described formamidopyrimidine-DNA glycosylase (12), since the two

activities co-purify at a constant ratio. The relative importance of this enzyme in DNA repair after ionizing radiation should now be evaluated by genetic methods.

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