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DEMETHYLATION OF O^6-METHYLGUANINE IN A SYNTHETIC DNA POLYMER BY AN INDUCIBLE ACTIVITY IN *ESCHERICHIA COLI*[1]

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Summary: O^6-Methyl[8-^{3}H]deoxyguanosine in a synthetic DNA polymer, poly(dC,dG, m^6dG), is demethylated by cell-free extracts of *Escherichia coli* B/r adapted by exposure to *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, as shown by the appearance of ^{3}H-labeled deoxyguanosine in hydrolysates of the recovered DNA. The demethylating activity could not be detected in extracts of nonadapted *E. coli*. These results provide direct evidence that a previously described inducible repair activity in *E. coli* acts by demethylating O^6-methylguanine at the DNA level.

O^6-Alkylguanine is believed to be the main mutagenic and carcinogenic lesion produced in DNA by simple alkylating agents (1,2). The capacity of organisms to repair such lesions and the mechanisms of repair involved are consequently of great interest.

A model system for repair studies is provided by the 'adaptive response' (3) of *Escherichia coli* grown in the presence of low levels of alkylating agents such as *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG). The adapted cells acquire resistance to the mutagenic and lethal effects of such agents, and this phenomenon has been correlated with the induction of rapid repair of O^6-alkylguanine lesions (4,5). Extracts of such adapted cells are also capable of removing O^6-methylguanine lesions from DNA in a cell-free system (6). However, the mechanism of the repair process has not been determined.

Previous studies of O^6-methylguanine repair, both *in vivo* and in cell-free systems, have involved DNA substrates in which O^6-methylguanine was

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produced by treatment with radioactively labeled methylating agents such as N-[^{3}H]methyl-N-nitrosourea. Because the O^6-methylguanine contained in these substrates is labeled in the O^6-methyl group, loss of radioactivity from the DNA could be due to removal of the O^6-methyl group by a demethylase, excision of O^6-methylguanine by an N-glycosylase, or excision of O^6-methyl-dGMP by a specific nuclease activity. Initial studies by Karran et al. (6) indicated that none of these mechanisms was operative in adapted E. coli but that O^6-methylguanine was altered in situ in some unknown way. However, more recent work by Olsson and Lindahl (7) suggests that the O^6-methyl group is transferred to a protein cysteine residue by a methyltransferase activity present in extracts of adapted E. coli cells.

In the present study we have used a synthetic DNA polymer, poly(dC,dG, m^{6}dG) containing O^6-methyl[8-^{3}H]guanine, to demonstrate the in situ demethylation of the alkylated base by an activity contained in a crude extract of adapted E. coli cells. Demethylation can be clearly shown to occur at the DNA level and quantitatively assayed by the formation of labeled deoxyguanosine in this substrate. The assay is uncomplicated by the presence of other labeled methylation products (8) which are formed by reaction of DNA with radioactive methylating agents. The results of preliminary studies of the rate and extent of demethylation by the extract are also reported.

MATERIALS AND METHODS

[8-^{3}H]Deoxyguanosine (1.4 Ci/mmol) was obtained from ICN Pharmaceuticals. Unlabeled deoxyguanosine was obtained from Boehringer Mannheim Corp., and nucleoside triphosphates were from P-L Biochemicals. Terminal deoxynucleotidyl transferase, pancreatic DNase, venom phosphodiesterase, and alkaline phosphatase were obtained from Worthington Biochemical Corp.

Unlabeled O^6-methyldeoxyguanosine (m^{6}dG) was prepared by the method of Farmer et al. (9). O^6-Methyl[8-^{3}H]deoxyguanosine 5'-triphosphate ([^{3}H]m^{6}dGTP) was prepared from [8-^{3}H]deoxyguanosine (0.2 mmol, sp. act. ~15 mCi/mmol) essentially by the procedure described by Abbott et al. (10). The overall yield of triphosphate was 8%, and the specific activity was 11 cpm/pmol.

Poly(dC,dG,[^{3}H]m^{6}dG) was prepared by polymerization of nucleotides with terminal deoxynucleotidyl transferase (11). The polymerization mixture (0.5 ml) contained 1 mM dCTP, 0.5 mM dGTP and 0.5 mM [^{3}H]m^{6}dGTP (11 cpm/pmol), 200 mM potassium cacodylate buffer (pH 7.0), 40 mM KCl, 1 mM $CoCl_2$, 1 mM 2-mercaptoethanol, 10 μM $p(dT)_3$ as initiator, and approximately 50 units of terminal transferase. Mixtures were incubated at 37° for 24 hr and extracted with phenol. The DNA polymer was then purified by column chromatography on Sephadex G-50. Approximately 35% of the [^{3}H]m^{6}dGTP was incorporated into the polymer.

E. coli B/r was grown and adapted by exposure to MNNG (1 μg/ml for 90 min) as described by Jeggo et al. (3). Extracts were prepared from adapted or nonadapted cells as described by Karran et al. (6), adjusted to a concentration of 25 mg of protein per ml, and stored in small aliquots at -80°.

Reaction mixtures (200 µl) of cell extracts and poly(dC,dG,[^{3}H]m^{6}dG) contained 70 mM HEPES-KOH (pH 7.8, 1 mM dithiothreitol, 5 mM EDTA, 50 µM spermidine, and poly(dG,dC,[^{3}H]m^{6}dG) and cell extract protein as described. After incubation the reaction mixtures were extracted with phenol or phenol/$CHCl_3$ (1:1), and the DNA was precipitated from the aqueous layer by addition of cold ethanol in the presence of 0.3 M sodium acetate. After centrifugation at 10,000 x g for 10 min, the supernatant was discarded and the precipitated DNA was dried for a few minutes in vacuo. Of the added radioactivity, 80-95% was routinely recovered in the precipitated DNA.

The DNA was enzymatically hydrolyzed to nucleosides by treatment at 37° for 1 hr with 50 µg of pancreatic DNase in 20 µl containing 15 mM ammonium acetate and 30 mM magnesium acetate (pH 7.2), followed by addition of 25 µl of 200 mM ammonium acetate (pH 8.8), 20 µl of venom phosphodiesterase (10 µg/µl), and 5 µl of bacterial alkaline phosphatase (10 µg/µl), and incubation at 45° for 3 hr.

Aliquots of the hydrolysis mixture plus unlabeled markers (dG and m^{6}dG) were injected directly into an Aminex A-6 column (24 x 0.63 cm) and eluted by 0.1 M ammonium borate (pH 8.0) at a flow rate of 0.31 ml/min and a column temperature of 50° (12). The effluent was monitored at 260 and 280 nm. Fractions (0.6 ml) were collected every 2 min and mixed with ACS scintillation solvent (Amersham) for the determination of radioactivity in a Beckman scintillation spectrometer. Radioactivity which co-eluted with marker dG was further characterized after evaporation of the appropriate column fractions with methanol to remove ammonium borate. The residue was dissolved in water and injected into a second Aminex A-6 column eluted with 0.4 M ammonium formate (pH 4.5). Fractions were collected and assayed for radioactivity as before.

RESULTS AND DISCUSSION

The incubation of poly(dC,dG,[^{3}H]m^{6}dG) with adapted cell extract resulted in partial conversion of [^{3}H]m^{6}dG residues to [^{3}H]dG, as shown by chromatographic analysis of enzymatic hydrolysates of the recovered DNA polymer (Fig. 1). The identification of [^{3}H]dG was supported by its co-elution with unlabeled dG in a second chromatographic system. No [^{3}H]dG was detected in hydrolysates of poly(dC,dG,[^{3}H]m^{6}dG) which had been incubated with an equal concentration of nonadapted cell extract.

Preliminary studies indicate that the adapted cell extract has a limited capacity to carry out the demethylation reaction. Although the rate of demethylation of substrate (240 pmol) in the presence of 4 mg of extract protein is initially high, it rapidly levels off to yield less than 40% conversion of m^{6}dG to dG after 1 hr (Fig. 2). This limited extent of demethylation is apparently not due to the inaccessibility of part of the m^{6}dG residues in the substrate, since more than 60% conversion could be obtained in the presence of the same amount of extract by lowering the concentration of the substrate by 50%. Furthermore, it does not appear to be due to deactivation of the induced activity by reaction conditions, since preincubation of a reaction mixture containing 2 mg of protein at 37° for 10 min before addition

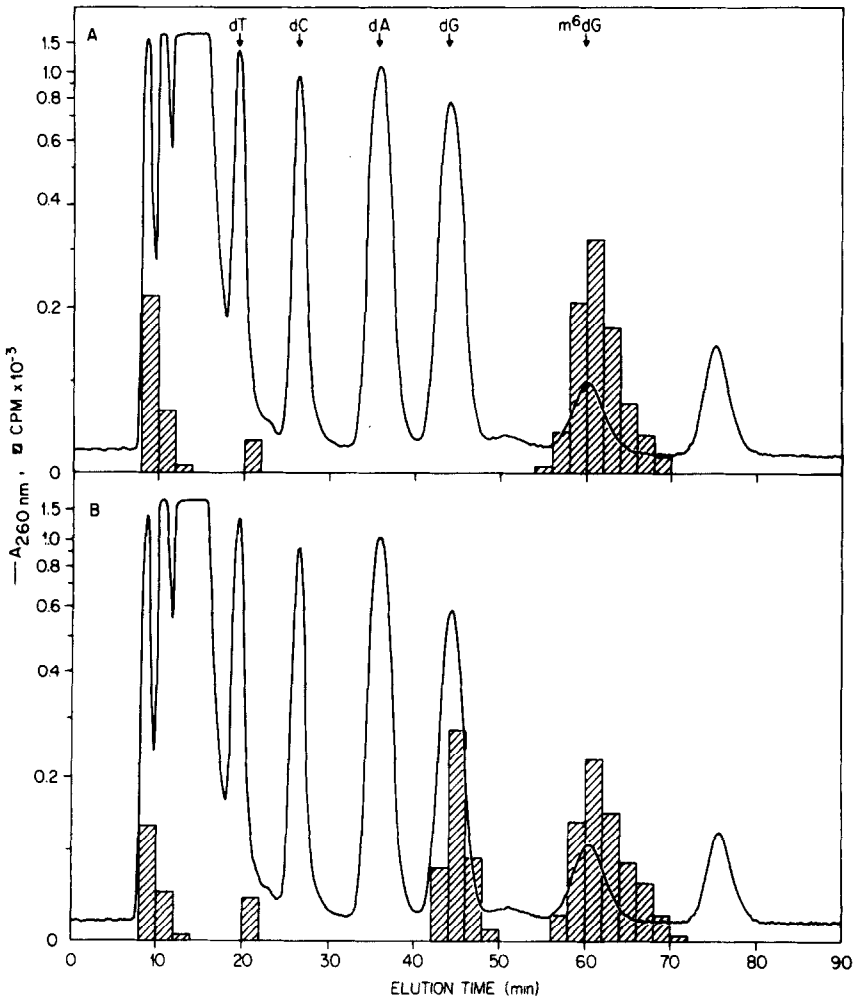
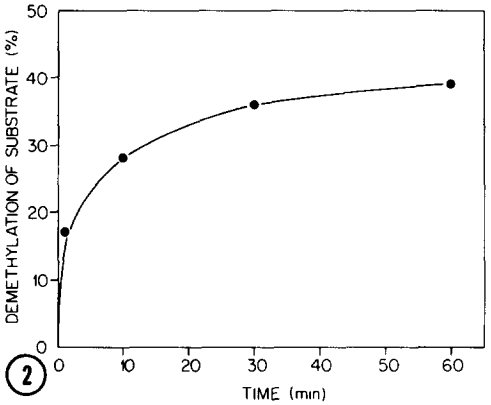


Fig. 1. Chromatographic analysis of enzymatic hydrolysates of recovered poly(dC,dG,[8-^{3}H]m^{6}dG) after incubation for 1 hr at 37° with extract from (A) nonadapted and (B) adapted cells. Each incubation contained 240 pmol (2600 cpm) of [^{3}H]m^{6}dG (contained in polymer) and 4 mg of cell extract protein. Background radioactivity of 22 cpm was subtracted from each fraction. The normal unlabeled deoxynucleosides arose from hydrolysis of endogenous DNA contained in cell extracts.

of substrate had no effect compared with an identical reaction which was not preincubated. Both reactions were incubated with 240 pmol substrate for 10 min and resulted in 21% conversion of m^{6}dG to dG.

The extent of demethylation during 1-hr incubations was linearly dependent on the amount of protein added to the reaction mixture up to 3 mg (Fig. 3). The amount of m^{6}dG converted to dG per mg of protein was equal to approximately 32 pmol/mg.



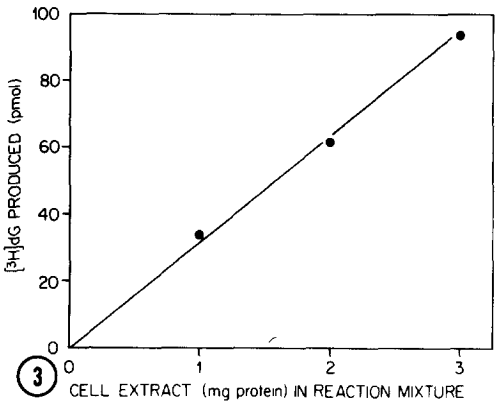


Fig. 2. Time course of demethylation of O^6-methylguanine in poly(dC,dG, [8-^{3}H]m^6dG) with 4 mg of adapted E. coli cell protein at 37°. Each time point was obtained from a separate incubation containing 240 pmol of m^6dG (contained in polymer). The percentage of O^6-methylguanine demethylated was calculated from the relative amounts of [^{3}H]dG and [^{3}H]m^6dG in hydrolysates of recovered DNA.

Fig. 3. Dependence of demethylation of O^6-methylguanine in poly(dC,dG, [8-^{3}H]m^6dG) on the amount of adapted E. coli cell extract in reaction mixtures. Each reaction mixture contained 240 pmol of m^6dG (contained in polymer) and was incubated for 1 hr at 37° with the amount of extract shown. The total amount of [^{3}H]dG produced was calculated by multiplying 240 pmol by the fraction of m^6dG converted to dG in recovered substrate.

The rate of demethylation was significantly reduced at lower temperatures. When reaction mixtures containing 2 mg of protein were incubated with substrate for 1 hr at 0 or 20°, the conversion of m^6dG to dG was reduced by 57 or 11%, respectively, compared with the conversion at 37°.

The results of our studies are consistent with the proposal of Robins and Cairns (5) that each molecule of induced repair agent in adapted E. coli can repair only one O^6-methylguanine residue in DNA in vivo. These authors calculated that fully adapted cells of E. coli AB1157 (4) each contain approximately 3000 molecules of the repair agent. Assuming that each repair molecule is used only once, we have calculated approximately 3100 molecules per cell in adapted E. coli B/r, based on the approximate number of cells harvested per mg of extracted protein (6.4×10^9 cells/mg) and on the value of 32 pmol (2.0×10^{13} molecules) of [^{3}H]dG produced per mg of protein.

The limited capacity of adapted cells and cell extracts to repair O^6-methylguanine in DNA could alternatively be due to limiting amounts of an acceptor molecule to which the methyl group is transferred by an induced enzyme. The determination of the exact mechanism of the demethylation process will require the use of purified activities.

These experiments demonstrate the advantages of using a synthetic DNA substrate containing ring-labeled O^6-methylguanine for studies of the demethylation reaction. Retention of the labeled demethylation product, [^{3}H]dG, in the DNA along with unreacted [^{3}H]m^6dG allows a quantitative assay of demethylation which is independent of the efficiency of substrate recovery and hydrolysis. Such substrates should be very useful in the purification and characterization of repair activities from E. coli as well as other systems such as rat liver (13,14) which are capable of repairing O^6-methylguanine lesions in DNA.

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