The EMBO Journal vol.3 no.3 pp.545–550, 1984

Inducible repair of O-alkylated DNA pyrimidines in *Escherichia coli*

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Communicated by T. Lindahl

The three miscoding alkylated pyrimidines O^2-methylcytosine, O^2-methylthymine and O^4-methylthymine are specifically recognized by *Escherichia coli* DNA repair enzymes. The activities are induced as part of the adaptive response to alkylating agents. O^2-Methylcytosine and O^2-methylthymine are removed by a DNA glycosylase, the *alkA*$^+$ gene product, which also acts on N^3-methylated purines. O^4-Methylthymine is repaired by a methyltransferase, previously known to correct O^6-methylguanine by transfer of the methyl group to one of its own cysteine residues. It is proposed that certain common structural features of the various methylated bases allow each of the two inducible repair enzymes to recognize and remove several different kinds of lesions from alkylated DNA.

Key words: chemical mutagenesis/N-nitroso compounds/adaptive response/O^2-methylcytosine/O^4-methylthymine

Introduction

Mutagenesis induced by direct-acting methylating and ethylating agents appears to be largely dependent on the ability of these compounds to alkylate DNA bases at oxygen atoms (Singer, 1976). The major O-alkylated base derivative in DNA treated with agents such as N-methyl-N-nitrosourea (MNU) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) is O^6-methylguanine (O^6MeGua), and the occurrence and persistence of this altered base in DNA *in vivo* has been directly correlated with the mutagenic and carcinogenic effects of the agents (Beranek *et al.*, 1983; Bartsch *et al.*, 1983). O^6-Methylguanine appears to form base pairs preferentially with thymine rather than cytosine during DNA replication, and the alteration gives rise to transition mutations (Abbott and Saffhill, 1979). While no O-alkylated purines other than O^6-methylguanine can be generated in DNA, three different O-alkylated pyrimidines are produced after exposure to MNU, MNNG or analogous ethylating agents. These base derivatives, O^2-methylcytosine (O^2MeCyt), O^2-methylthymine (O^2MeThy) and O^4-methylthymine (O^4MeThy) (Figure 1), occur at lower levels than O^6-methylguanine (Lawley *et al.*, 1973; Singer, 1976; Jensen, 1978). However, they have recently been shown to exhibit strong miscoding properties (Hall and Saffhill, 1983; Singer *et al.*, 1983) and would therefore be expected to contribute to alkylation mutagenesis.

Bacteria, as well as higher cells, have the ability to repair several forms of DNA alkylation damage. Thus, N-alkylated purines such as 3-methyladenine (N^3MeAde) and 3-methylguanine (N^3MeGua) are removed by DNA glycosylases, while O^6-methylguanine is corrected by transfer of the methyl group to a cysteine residue in a protein. In *Escherichia coli*, these repair functions are inducible during the adaptive response to alkylating agents (Cairns *et al.*, 1981; Lindahl, 1982). The persistence and possible repair of alkylated pyrimidines in DNA has received less attention. However, studies on the fates of O-ethylated pyrimidine residues in the DNA of mammalian cells have indicated that active repair of such lesions occurs (Bodell *et al.*, 1979; Singer *et al.*, 1981). In the present work, the *E. coli* DNA repair functions that recognize O-alkylated pyrimidines have been characterized.

Results

Release of O^2-methyl pyrimidines by a DNA glycosylase activity

Pyrimidines methylated at exocyclic oxygen or ring nitrogen atoms are minor reaction products in DNA treated with N-nitroso compounds such as MNU or MNNG. Consequently, their detection and identification requires access to large amounts of radioactively labelled methylated DNA and separation systems of high resolution. In a search for DNA repair enzymes that might act on alkylated pyrimidines, we initially investigated if any such residues in [^{3}H]MNU-treated DNA could be released in free form by a DNA glycosylase activity present in crude cell extracts from *E. coli*. After incubation of appropriate reaction mixtures, an initial separation between methylated purines and pyrimidines was carried out using paper chromatography. The individual methylated pyrimidines were then resolved by h.p.l.c. analysis.

When methylated *Micrococcus luteus* DNA was incubated with an extract from *E. coli* F26 cells which had been induced

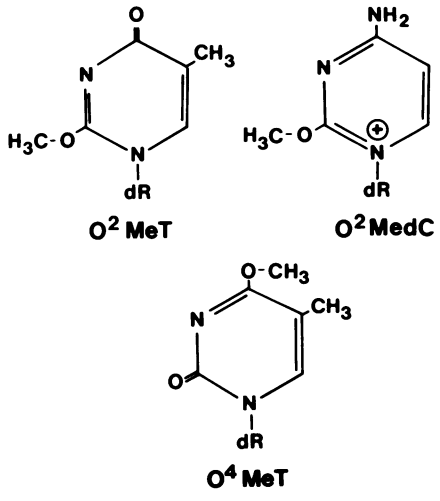

Fig. 1. The three possible O-methylated thymine and cytosine derivatives in DNA.

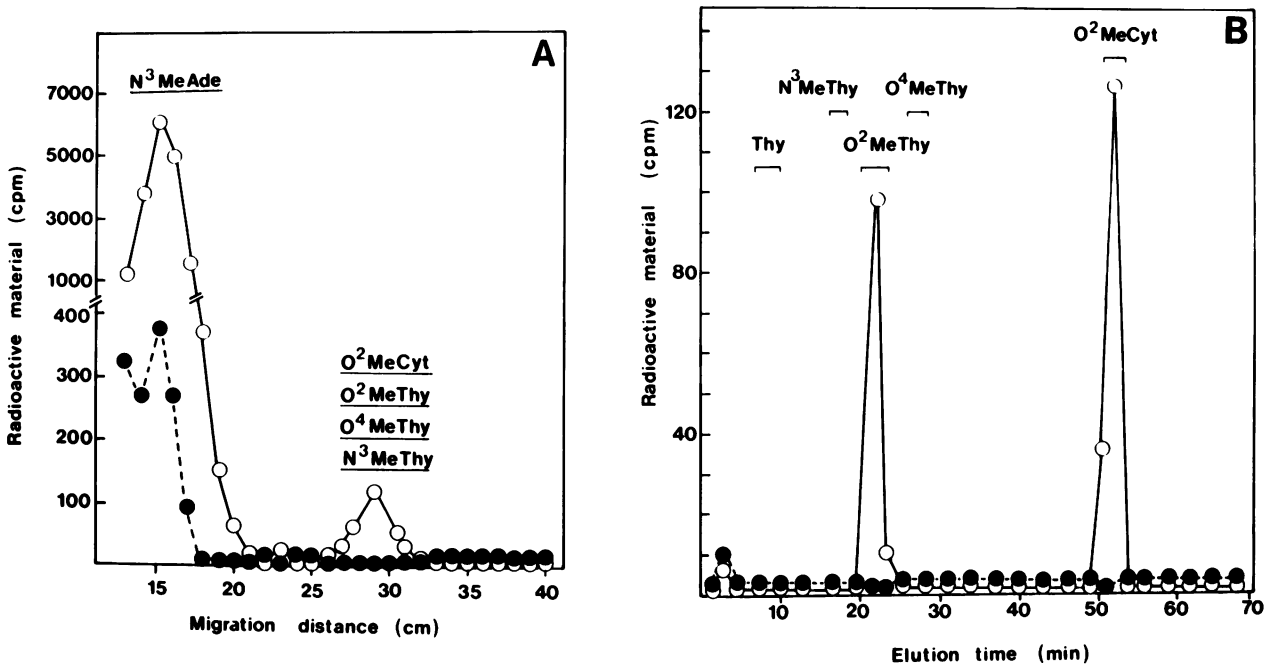

Fig. 2. Analysis of O-alkylated pyrimidines released in free form from alkylated *M. luteus* DNA by a crude cell extract from *E. coli*, induced for the adaptive response. **(A)** Paper chromatography of ethanol-soluble material from standard reaction mixtures. O-Methylated pyrimidines co-chromatographed and were resolved from N^3MeAde, which was liberated both by DNA glycosylase action and (to a much smaller extent) by non-enzymatic hydrolysis. The minor alkylation product N^3MeGua co-chromatographed with N^3MeAde. Open circles: reaction mixture with cell extract (100 μg). Closed circles: control without cell extract. **(B)** H.p.l.c. analysis of enzymatically liberated O-alkylated pyrimidines (see Figure 2A). Symbols as above.

for the adaptive response to alkylating agents by MNNG treatment (Cairns *et al.*, 1981), 10% of the total radioactive material was recovered in ethanol-soluble form. Analysis of the released products by paper chromatography showed that the main component was 3-methyladenine. However, a small, fast-moving peak of radioactive material (R_f 0.8) which contained 0.1% of the total radioactivity was also observed (Figure 2A). It co-chromatographed with the authentic O^2MeCyt, O^2MeThy, O^4MeThy, and N^3MeThy markers. (N^3MeCyt had a R_f value of 0.35 and was not investigated.)

The released, rapidly migrating material comprised ~60% of the total O-methylated pyrimidines initially present in the DNA. On subsequent h.p.l.c. analysis, two distinct peaks of radioactive material were observed. The smaller one had the retention time characteristic of O^2MeThy (22 min), while the second, larger peak co-eluted with authentic O^2MeCyt at 51 min (Figure 2B). No detectable amounts of radioactive material co-eluted with the N^3MeThy or O^4MeThy markers. The identities of the released compounds, O^2MeCyt and O^2MeThy, were confirmed in six additional h.p.l.c. systems. Furthermore, it was also observed that < 1% of the O^2MeCyt initially present in the alkylated DNA remained after incubation with the cell extract. Since the glycosyl bonds between these O^2-methylpyrimidines and deoxyribose in DNA are relatively stable at neutral pH and 37°C (Singer *et al.*, 1978), it is concluded that these bases were liberated enzymatically.

The relative extents of active release of O^2MeCyt and O^2MeThy from methylated DNA were investigated by incubation of reaction mixtures with increasing amounts of crude cell extract from adapted *E. coli* (Figure 3). The O^2MeCyt was liberated in preference to O^2MeThy. Thus, >85% of the O^2MeCyt was released before free O^2MeThy could be detected. However, with larger amounts of extract, both bases could be quantitatively removed from DNA.

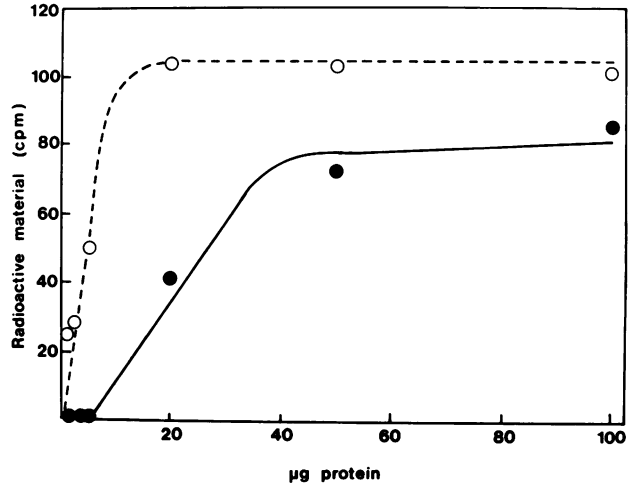

Fig. 3. Release of O^2-methylated pyrimidines from alkylated calf thymus DNA by varying amounts of a crude cell extract from adapted *E. coli*. Open circles show O^2MeCyt, and closed circles O^2MeThy.

Identification of the DNA glycosylase

The DNA glycosylase activity for O^2-methylated pyrimidines was found to be induced as part of the adaptive response to alkylating agents. Thus, extracts from adapted *E. coli* F26 exhibited levels of O^2-methylpyrimidine-DNA glycosylase activity 20-fold higher than extracts of non-adapted cells (Figure 4). Furthermore, extracts of *E. coli* mutants (*adc*) expressing the adaptive response in a constitutive fashion (Sedgwick and Robins, 1980) contained 10-fold higher activity than extracts of non-adapted wild-type cells. Other mutants (*ada*), in which the adaptive response is not inducible, showed the same low level of enzyme activity in cell extracts both before and after attempted induction of the adaptive response (Figure 4).

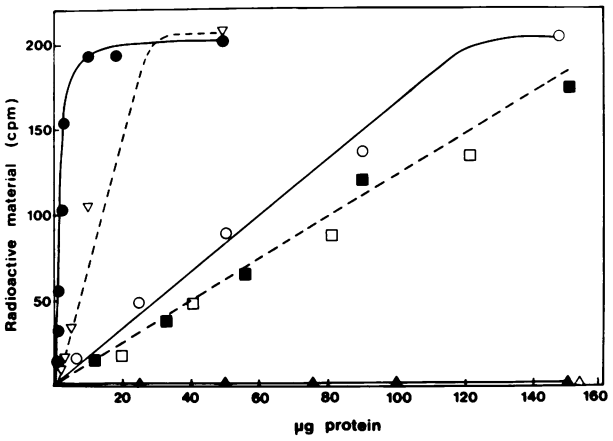

Fig. 4. Release of methylpyrimidines (O^2MeCyt and O^2MeThy) from alkylated *M. luteus* DNA by crude cell extracts from different *E. coli* strains. Closed symbols show extracts from bacteria induced for the adaptive response, while open symbols represent unadapted cells. Circles: strain F26 (wild-type). Squares: strain BS23 (*ada*). Inverted triangles: strain BS31 (*adc*). Triangles: strain MS23 (*alkA*).

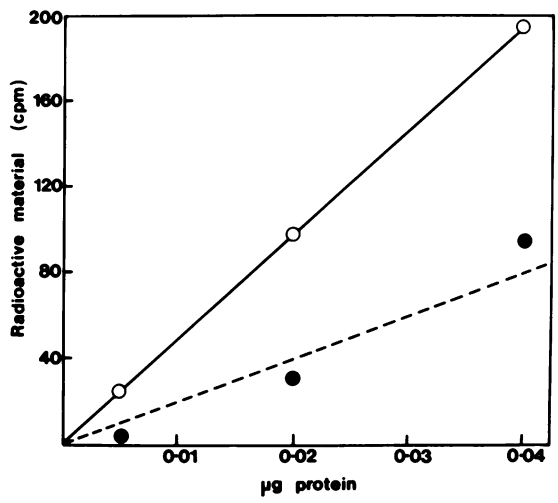

Fig. 5. Release of O^2-methylated pyrimidines (open circles) and N^3-methylated purines (closed circles) from alkylated *M. luteus* DNA by a 1000-fold purified preparation of *E. coli* 3-methyladenine-DNA glycosylase II.

These data establish that the release of O^2-methylpyrimidines from alkylated DNA cannot be due to most of the previously characterized DNA glycosylases, since they are not induced during the adaptive response (Karran *et al.*, 1982). However, one such activity was known to be present at higher levels in adapted cells. This is the 3-methyladenine-DNA glycosylase II, which efficiently catalyzes the release of the N-methylated purines, N^3MeAde and N^3MeGua, from alkylated DNA (Karran *et al.*, 1982). The structural gene for this inducible DNA glycosylase is *alkA*$^+$, located at 43 min on the standard *E. coli* K-12 genetic map (Evensen and Seeberg, 1982; Yamamoto *et al.*, 1983). To investigate if the inducible DNA glycosylase activities for O^2MeCyt and O^2MeThy were also associated with the *alkA*$^+$ gene product, cell extracts from two independently isolated *alkA* mutants were assayed for release of O^2-methylpyrimidines. No such activity could be detected in extracts from these mutants, either before or after attempted adaptation (Figure 4). As a control, another repair enzyme induced during the adaptive response, the O^6-methylguanine-DNA methyltransferase, was shown to be present in expected amounts in the cell extracts from the adapted *alkA* strains. These data show that the newly detected DNA glycosylase activities for O^2MeCyt and O^2MeThy may be ascribed to the *alkA*$^+$ gene product, that is, the 3-methyladenine-DNA glycosylase II.

The association of glycosylase activities for O^2-methylated pyrimidines and N^3-methylated purines with a single enzyme was confirmed by purification of the 3-methyladenine-DNA glycosylase II by the five-step procedure of Thomas *et al.* (1982). The activities of N^3MeAde, N^3MeGua, O^2MeCyt, and O^2MeThy co-chromatographed and were consequently all enriched to the same extent in a 1000-fold purified enzyme preparation. The O^2-methylpyrimidines were released at least 2-fold more effectively than N^3-methylpurines by the enzyme (Figure 5).

Repair of O^4-methylthymine by a DNA methyltransferase

No DNA glycosylase activity for O^4MeThy was observed in any of the experiments described above (Figure 2B). An alternative mechanism for repair of this lesion could involve a DNA methyl group transfer reaction, similar to that which corrects O^6MeGua in DNA (Lindahl *et al.*, 1982). To investigate this possibility, we analyzed the methylated residues remaining in alkylated DNA after incubation with cell extracts. Ethanol precipitates of DNA from reaction mixtures were redissolved and degraded to deoxynucleosides by digestion with DNase I, phosphodiesterase, and alkaline phosphatase. [The spontaneous demethylation of O^4MeThy that occurs at low pH (Singer *et al.*, 1978) precluded the use of acid hydrolysis.] After the enzymatic hydrolysis, separation of O^4-methylthymidine (O^4MeT) from all other alkylated deoxynucleosides was achieved in two steps. First, methylated pyrimidine deoxynucleosides were separated from methylated purine deoxynucleosides by paper chromatography. O^4MeT and 3-methylthymidine (N^3MeT) migrated together with an R_f of 0.8, while O^2-methylthymidine and O^6-methyldeoxyguanosine migrated with R_f values of 0.68 and 0.65, and all other detectable alkylated deoxynucleosides had R_f values of <0.5. In the second step, radioactive material which co-migrated with authentic O^4MeT and N^3MeT was eluted from the paper, and the methylated deoxynucleosides resolved by h.p.l.c.

Incubation of alkylated DNA with extracts of non-adapted *E. coli* cells had no detectable effect upon the subsequent recovery of O^4MeT and N^3MeT. In contrast, when extracts of adapted *E. coli* cells were employed, only 10–15% of the O^4MeT initially present could be detected, whereas 90–100% of the N^3MeT remained (data not shown). Since neither of these alkylated pyrimidines was released in free form (Figure 2B), the results indicate that O^4MeT residues are corrected by an inducible mechanism that does not involve a DNA glycosylase, while N^3MeT residues are not repaired, at least under our *in vitro* conditions.

These results are quite similar to the initial observations on the removal of O^6-methylguanine from DNA by extracts from adapted *E. coli* (Karran *et al.*, 1979). In that case, a protein was subsequently isolated which transferred the methyl group from the O^6 position of guanine to one of its own cysteine residues (Olsson and Lindahl, 1980). In order to investigate if this activity, O^6-methylguanine-DNA methyltransferase, could also remove a methyl group from the O^4 position of a thymine residue, the remaining O^4MeT was measured after incubation of alkylated DNA with the

homogeneous enzyme (Demple *et al.*, 1982). The amount of methyltransferase employed in this experiment was ~2-fold in excess of the quantity required to correct all the O^6MeGua residues present in the DNA substrate. The pure transferase efficiently repaired O^4MeT residues, but not N^3MeT residues (Figure 6). Thus, O^6-methylguanine-DNA methyltransferase acts on both O^6-methylguanine and O^4-methylthymine in alkylated DNA.

O^6-Methylguanine in DNA is corrected very rapidly by the transferase, in ~0.5 s under standard reaction conditions at 37°C (Lindahl *et al.*, 1982). In contrast, substrate analogues such as O^6-ethylguanine and O^6-hydroxyethylguanine are dealkylated 100–500 times more slowly, and therefore require incubation periods of at least 10 min to approach stoichiometric repair in standard assays (Robins *et al.* 1983). Identical amounts of methyl group transfer from O^4MeThy were observed after incubation for either 10 s, 2 min or 15 min. This residue is thus corrected rapidly and efficiently by the transferase. However, in comparisons between the extents of correction (at limiting enzyme concentrations) of the two O-alkylated bases in DNA, O^6MeGua and O^4MeThy, we repeatedly observed that repair of O^6MeGua was more efficient. Thus, after incubation of alkylated DNA with an extract from adapted cells, 15% of the O^4-methylthymine initially present, but < 1% of the O^6-methylguanine, remained in the hydrolysate. This might reflect a preference by the transferase for one of its two substrates, analogous to that observed for the 3-methyladenine-DNA glycosylase II with regard to O^2MeCyt and O^2MeThy (Figure 3).

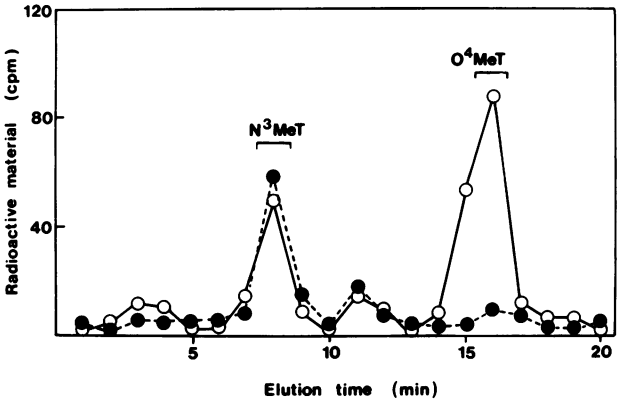

Fig. 6. H.p.l.c. analysis of O^4-methylthymidine in alkylated DNA after incubation with O^6-methylguanine-DNA methyltransferase. 60 μg calf thymus DNA (3 x 10^5 c.p.m.) were incubated with 26 units of the enzyme for 10 s at 37°C. The DNA was then enzymatically digested to deoxynucleosides for analysis. Closed circles: reaction mixture with enzyme. Open circles: control without enzyme.

As shown above, O^2MeCyt and O^2MeThy are removed efficiently from alkylated DNA by a glycosylase. This does not preclude the possibility that O^2-methylated pyrimidines might also be repaired to some extent by the methyl transfer pathway. To study this point, the amounts of O^2MeCyt in alkylated DNA were analysed before and after incubation with an excess of the homogeneous O^6-methylguanine-DNA methyltransferase. No detectable repair of O^2MeCyt by this enzyme was observed. (O^2MeThy was not analysed, because in contrast to O^2MeCyt it cannot be quantitatively released from DNA by neutral thermal hydrolysis). Moreover, no correction of N^7MeGua or N^3MeAde by the transferase was detected, in agreement with previous results (Lindahl, 1982), while O^6MeGua and O^4MeThy were quantitatively repaired.

Discussion

The mutagenic effects of simple alkylating agents may be ascribed to the generation of miscoding bases in DNA. The majority of these adducts are removed by various DNA repair pathways and the fates of alkylated purines (which are the major reaction products) have been investigated and characterized in many laboratories. In contrast, no information has been available on the mechanisms of repair of alkylated pyrimidines. We have now shown that *E. coli* contain enzyme activities which remove O-alkylated pyrimidines from DNA. Since these lesions are miscoding, the enzymes involved would be expected to participate in the antimutagenic defence of the organism. Bacterial mutant strains defective in such enzymes should consequently exhibit increased susceptibility to mutagenesis by alkylating agents. However, our findings suggest that specific mutagenic effects by O-alkylated pyrimidines *in vivo* may be difficult to demonstrate, because the activities recognizing such lesions are not functions of previously unknown enzymes. Instead, they represent activities of two inducible repair enzymes which also serve in the removal of methylated purines from DNA.

One of these enzymes, the product of the *alkA*$^+$ gene, is a DNA glycosylase that exhibits a capacity to remove a variety of different methylated bases from DNA. This broad substrate specificity was an unexpected finding for a DNA glycosylase, because the first few known enzymes of this class appeared to be highly specific, each recognizing a single base lesion. For example, the uracil-DNA glycosylase can remove uracil (and at a slow rate 5-fluorouracil) from DNA, but not

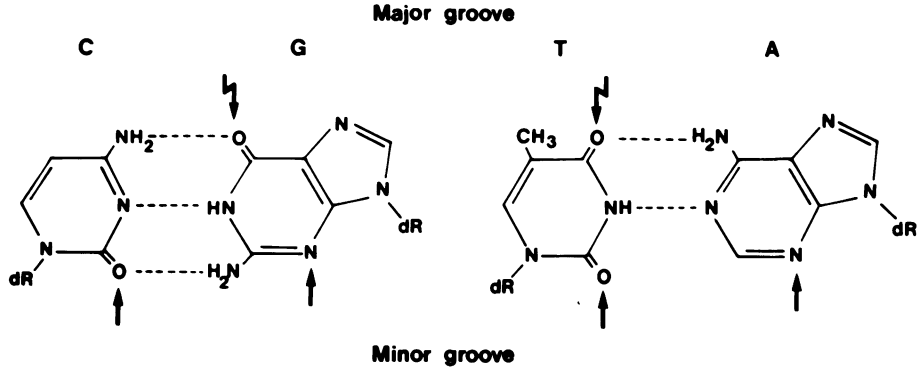

Fig. 7. Schematic diagram of recognition sites of 3-methyladenine-DNA glycosylase II (straight arrows) and O^6-methylguanine-DNA methyltransferase (hooked arrows).

any other known base derivatives (Lindahl *et al.*, 1977; Warner and Rockstroh, 1980). Similarly, hypoxanthine-DNA glycosylase does not remove xanthine or methylated purines from DNA (Karran and Lindahl, 1978). With regard to alkylation damage, the constitutively expressed *tag* gene product, 3-methyladenine-DNA glycosylase I, liberates efficiently the major killing lesion 3-methyladenine (and its analogue 3-ethyladenine) from DNA, but not similar derivatives such as 3-methylguanine or 7-methyladenine (Karran *et al.*, 1982; Thomas *et al.*, 1982). These DNA glycosylases appear to have evolved to recognize and remove a unique and important type of altered base residue. The DNA glycosylase investigated here seems different in that it can remove such structurally divergent compounds as purines methylated on a ring nitrogen or pyrimidines methylated on an exocyclic oxygen. Thus, the enzyme presumably recognizes a specific structural feature absent in the naturally occurring DNA base residues, rather than a specific altered base. In the present case, it is conspicuous that the methyl groups of 3-methyladenine, 3-methylguanine, O^2-methylcytosine and O^2-methylthymine all protrude into the minor groove of the DNA double helix (Figure 7). This groove is normally free from such groups, because the methyl groups of the naturally occurring bases thymine, 5-methylcytosine, and N^6-methyladenine are all located in the major groove. It was first suggested by Lawley and Warren (1976) that rapid repair of 3-methyladenine and 3-methylguanine might be necessary because methylation of purines at the N^3 site could block the template function of DNA. This concept is easily expanded to include the O^2-alkylpyrimidines. Thus, we propose that the inducible product of the *alkA*$^+$ gene functions by patrolling the minor groove of the DNA double helix, removing potentially cytotoxic and mutagenic methyl groups. In agreement with this model, *E. coli* mutants deficient either in the structural *alkA* gene, or in the control *ada* gene necessary for its induction, exhibit greatly increased sensitivity to killing by methylating agents (Yamamoto *et al.*, 1983). It is noteworthy that in space-filling models of DNA, O^2 positions of pyrimidines appear somewhat more accessible than N^3 positions of purines (Jensen and Reed, 1978), which might explain the more rapid removal of O^2-methylpyrimidines by the enzyme (Figure 5). However, the altered charge distributions produced by methylation of the various bases might also affect their relative abilities to serve as substrates. The *alkA*$^+$ gene product (3-methyladenine-DNA glycosylase II) does not contain an intrinsic nuclease activity for apurinic and apyrimidinic sites (Thomas *et al.*, 1982), but correction of the lesions would nevertheless be expected to occur by the base excision-repair pathway.

The methyl group of O^4-methylthymine protrudes into the DNA major groove, and this base is not removed by a DNA glycosylase. Instead, it is recognized and repaired by the inducible methyltransferase that also acts on O^6-methylguanine in DNA. Ahmmed and Laval (1984) and Schendel *et al.* (1983) have also observed that O^4MeThy residues in MNU-treated poly d(A-T) are repaired by crude cell extracts of adapted *E. coli,* through a methyl transfer mechanism, accompanied by the appearance of protein methyl groups. Although the transferase recognizes the two dissimilar adducts O^6MeGua and O^4MeThy, the enzyme clearly cannot act by removing all methyl groups in the major DNA groove, since thymine, 5-methylcytosine and N^6-methyladenine are not substrates. Further, the transferase cannot simply be acting on any methoxy group in DNA, because it was unable to abstract a methyl group from O^2-methylcytosine. In conclusion, it would appear that the transferase is able to remove methyl groups bound to base oxygen atoms within the major groove of the double helix (Figure 7).

The multi-subunit *uvrABC*$^+$ excision nuclease of *E. coli* (Sancar and Rupp, 1983) is known to act on a variety of dissimilar, bulky chemical adducts in addition to cyclobutane pyrimidine dimers in DNA. However, in that case, a major structural distortion of the double helix, rather than specifically localized altered sites, appears to be the unifying feature of enzyme recognition. Chemical mutagens such as N-nitroso compounds introduce many different lesions in DNA, some of which appear only in small quantities. Several of these minor lesions would be expected to show miscoding or cytotoxic effects. Nevertheless, the existence of individual DNA glycosylases or methyltransferases for each such lesion seems unlikely, because the number of repair enzymes postulated would be inordinately large. The recognition of a distinct type of covalent modification rather than a specific alkylated base residue, as observed for the enzymes investigated here, offers a solution to this dilemma. Thus, certain repair enzymes could each remove a small group of similar lesions.

Materials and methods

Bacterial strains

The parental wild-type *E. coli* B strain F26, strain BS31 (*adc*) constitutive for the adaptive response to alkylating agents, and strain BS23 (*ada*) deficient in the adaptive response were obtained from Dr. B. Sedgwick. *E. coli* BK2114 (*tag*), deficient in the constitutively expressed 3-methyladenine-DNA glycosylase I, was supplied by Dr. E. Seeberg. The *E. coli* AB1157 derivative strain MS23 (*alkA*-1), deficient in the inducible 3-methyladenine-DNA glycosylase II, was obtained from Dr. M. Sekiguchi, while the *E. coli* W deletion mutant BD793 (△*alkA*) was supplied by Dr. B. Duncan. When required, cultures were adapted by treatment with 1 μg/ml MNNG for 1 h (Samson and Cairns, 1977). Crude cell extracts were prepared from bacteria after lysozyme-EDTA treatment (Conrad and Campbell, 1979). Extracts from adapted cells were assayed for O^6MeGua-DNA methyltransferase activity (Demple *et al.*, 1982) to verify that efficient induction had been achieved.

Alkylated DNA

Micrococcus luteus DNA, purchased from Miles Laboratories, Inc., contains variable amounts of neutral salts which reduce the efficiency of alkylation. It was therefore dissolved in 10 mM Tris-HCl (pH 7.5) and precipitated with two volumes of cold ethanol. For alkylation, 2 mg of this DNA was dissolved in 3 ml 10 mM sodium cacodylate, 1 mM EDTA (pH 7.1), mixed with 1 mCi [^{3}H]MNU in 1 ml ethanol (1.5 Ci/mmol, NEN Inc.) and incubated for 90 min at 37°C. After addition of 0.04 volumes of 5 M NaCl, ethanol precipitation and repeated washing in 80% ethanol, the alkylated DNA was dissolved in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5), dialysed extensively against 0.1 M NaCl, 1 mM tri-sodium citrate, 10 mM Tris-HCl (pH 7.5) and stored at −20°C. The specific activity of the alkylated *M. luteus* DNA preparations was ~8000 c.p.m./μg DNA. Calf thymus DNA (Worthington) was treated with [^{3}H]MNU in the same fashion and contained ~5000 c.p.m./μg DNA. From analysis of hydrolysates (see below), it was estimated that the alkylated *M. luteus* DNA contained 0.06% of its total radioactivity as O^2MeCyt, 0.04% O^2MeThy, 0.05% O^4MeThy and 0.02% N^3MeThy. Most of the alkylation occurred on purine residues (65% N^7MeGua, 9% N^3MeAde, 7% O^6MeGua, 1% N^3MeGua) and at phosphate residues.

Reference compounds

2,4-Dimethoxy-5-methylpyrimidine was prepared by treatment of thymine with phosphorus oxychloride and sodium methoxide (Schmidt-Nickels and Johnson, 1930). The modified pyrimidine base O^2MeThy was obtained by alkali treatment of 2,4-dimethoxy-5-methylpyrimidine (Wong and Fuchs, 1970). For synthesis of O^4MeThy, 2,4-dimethoxy-5-methylpyrimidine was treated with acetyl chloride and then bicarbonate, as described by Wong and Fuchs (1970) except that the final chloroform extract was extracted with 3 volumes of water and the aqueous phase recovered. N^3MeThy was synthesized by treatment of thymine with trimethylphosphate (Yamauchi *et al.*, 1976). O^2MeCyt was made by alkylation of cytosine (Singer, 1976) with MNU (a gift from Dr. P.F. Swann) as follows: cytosine (1 mg) in 1 ml 0.2 M sodium cacodylate (pH 7.0), 1 mM EDTA, 25% dimethylsulfoxide, was treated with MNU (0.4 M) for 18 h at room temperature. Preparative chromatography on

Whatman 3MM paper in butanol:ethanol:water (80:10:25) yielded four u.v.-absorbing bands. The material in the fastest migrating band (R_f 0.8) which contained O^2MeCyt was eluted from the paper with water.

N^3MeAde and N^3MeGua were obtained from Fluka Ag. N^3MeCyt and N^7MeGua were purchased from Sigma. O^2-Methylthymidine and N^3-methylthymidine were prepared by treatment of thymidine with MNU in a manner similar to that described for preparation of O^2MeCyt. Preparative paper chromatography in the solvent system described above gave two u.v.-absorbing bands. The faster migrating band (R_f 0.8) contained a mixture of N^3MeT and O^4MeT, while the slower migrating band (R_f 0.68) contained O^2MeT. These compounds were eluted from the paper with water. O^4-Methylthymidine, used as reference, was a gift from Dr. P.D. Lawley. N^3MeT and O^4MeT were separated by h.p.l.c. O^6-Methyldeoxyguanosine was prepared in a similar manner to that described for isolation of O^2MeCyt. Methylation of deoxyguanosine with MNU produced several u.v.-absorbing derivatives that were resolved in the preparative paper chromatography system described above, and the fastest migrating band (R_f 0.65) contained O^6MedG. All markers synthesized were identified by their known u.v. absorption spectra, chromatographic properties, and sensitivities to acid-catalyzed demethylation (Singer *et al.*, 1978).

Enzymes and assays

3-Methyladenine-DNA glycosylase II was purified 1000-fold from *E. coli* BK2114 (*tag*) induced for the adaptive response, essentially by the procedure of Thomas *et al.* (1982). The most highly purified fraction exhibited five protein bands on SDS-polyacrylamide gel electrophoresis. The O^6-methylguanine-DNA methyltransferase employed was the physically homogeneous preparation described by Demple *et al.* (1982).

For DNA glycosylase assays, the standard reaction mixtures (100 μl) contained 0.07 M Hepes·KOH (pH 7.8), 2 mM dithiothreitol, 1 mM EDTA, 20 mM KCl, alkylated *M. luteus* DNA or calf thymus DNA (2.5 x 10^5 c.p.m.), and a limiting amount of enzyme. After 30 min at 37°C, the mixtures were chilled to 0°C and the DNA precipitated by addition of two volumes of cold ethanol. After 20 min at −20°C, the samples were centrifuged, and the supernatants recovered and supplemented with authentic methylated bases as markers for chromatographic analysis.

For DNA methyltransferase assays, 60 μg of alkylated calf thymus DNA (3 x 10^5 c.p.m.) was employed as substrate. The standard mixtures were the same as those described for the DNA glycosylase assays. The reaction was initiated by addition of either 26 units of O^6-MeGua-DNA methyltransferase, or crude cell extract (3 mg protein) from adapted or non-adapted cells. After incubation at 37°C for 15 min unless otherwise stated, the reaction was stopped by chilling the assay mixtures for 5 s in a dry ice-ethanol bath. The DNA was then precipitated by addition of 2 volumes of cold ethanol. After 20 min at −20°C, the samples were centrifuged, and the supernatants were discarded. The precipitates were re-dissolved in 500 μl of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 8 mM $MgCl_2$. Remaining protein was largely removed from the samples by extraction with one volume of chloroform-isoamyl alcohol (24:1), centrifugation, and recovery of the aqueous phase. The DNA was then digested to deoxynucleosides by incubation with 50 μg DNase I (Worthington) for 18 h at 37°C, followed by 100 μg snake venom phosphodiesterase (Sigma) for 12 h at 37°C. The authentic marker compounds O^4MeT and O^6MedG and 3.8 units of bacterial alkaline phosphatase (Sigma, heated at 95°C for 10 min prior to use to inactivate contaminating adenosine deaminase) were then added, and digestion continued for 4 h at 37°C. The samples were chilled, and the reagent proteins removed by precipitation with two volumes of cold ethanol. After centrifugation, the supernatants were recovered for chromatographic analysis.

Product analysis

A preliminary separation of O-alkylated pyrimidine bases and deoxynucleosides from alkylated purines was performed by paper chromatography on Whatman 3MM paper in butanol:ethanol:water (80:10:25) for 18 h. Papers were either cut into 1 cm strips which were eluted with water and analysed for radioactivity, or alkylated purine and pyrimidine derivatives were eluted from paper chromatograms and further analysed by reverse phase h.p.l.c. (Beranek *et al.*, 1980) using a Varian 5000 liquid chromatograph equipped with a Varian micropak column MCH-10. Separation of alkylated pyrimidine bases was performed at a flow rate of 1 ml/min with 10% methanol in water from time 0 to 35 min, a linear gradient from 10% to 65% methanol from 35 min to 45 min, and a further linear gradient to 100% methanol from 45 min to 70 min. Elution times of authentic markers in this system are as follows: Thy, 8 min; N^3MeThy, 17 min; O^2MeThy, 22 min, O^4MeThy, 26 min; O^2MeCyt, 51 min. For separation of alkylated pyrimidine deoxynucleosides, elution was performed with 20% methanol; N^3MeT had an elution time of 8 min, while O^4MeT was retained for 16 min.

O^2MeCyt remaining in DNA after treatment with crude cell extracts or purified enzymes was quantitated following hydrolysis at neutral pH (Singer *et al.*, 1978). Ethanol-precipitated DNA was dissolved in 100 μl 0.1 M sodium cacodylate (pH 7.0) and heated in sealed tubes at 100°C for 10 min. Samples were then chilled and DNA precipitated by addition of 0.04 volumes 5 M NaCl and 2 volumes cold ethanol. After centrifugation, the supernatants were recovered and analysed by chromatography.

Acknowledgements

We thank Drs. P.D. Lawley and P.F. Swann for generous gifts of reagents, Mr. M. Jones for skilful assistance in the preparation of reference compounds, and Drs. B. Duncan, B. Sedgwick, E. Seeberg and M. Sekiguchi for bacterial strains.

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Received on 21 November 1983