The Escherichia coli O\textsuperscript{6}-Methylguanine-DNA Methyltransferase Does Not Repair Promutagenic O\textsuperscript{6}-Methylguanine Residues When Present in Z-DNA*

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The repair of O\textsuperscript{6}-methylguanine present in N-methylnitrosourea (MNU)-treated alternating polynucleotides (MNU-poly(dG-dC)-poly(dG-dC) and MNU-poly(dG-me\textsuperscript{6}dC)-poly(dG-me\textsuperscript{6}dC)) was investigated using O\textsuperscript{6}-methylguanine-DNA methyltransferase purified from Escherichia coli. Both modified polynucleotides are equally good substrates for the DNA methyltransferase when they are in the B-form. The substrate properties of the MNU-treated polynucleotides do not differ from those of MNU-treated DNA. One of these modified polynucleotides, MNU-poly(dG-me\textsuperscript{6}dC)-poly(dG-me\textsuperscript{6}dC), can adopt the Z-conformation under physiological conditions. The conformational transition of the poly(dG-me\textsuperscript{6}dC)-poly(dG-me\textsuperscript{6}dC) from the B-form to the Z-form was monitored by the modification of its spectroscopic properties and by the specific binding of antibodies raised against Z-DNA. The O\textsuperscript{6}-methylguanine residues are repaired in MNU-poly(dG-me\textsuperscript{6}dC)-poly(dG-me\textsuperscript{6}dC) in B-form. At variance, the conversion of this template to the Z-form completely inhibits the repair of the O\textsuperscript{6}-methylguanine residues. The cooperative transition from the Z- to the B-form of MNU-poly(dG-me\textsuperscript{6}dC)-poly(dG-me\textsuperscript{6}dC), mediated by intercalating drugs such as ethidium bromide, restores the ability of MNU-poly(dG-me\textsuperscript{6}dC)-poly(dG-me\textsuperscript{6}dC) to be substrate for the transferase.

These results imply that the promutagenic DNA lesion O\textsuperscript{6}-methylguanine persists in Z-DNA fragments and suggest that DNA conformation modulates the extent of DNA repair and, as a result, plays an important role in determining the mutagenic potency of chemical carcinogens.

It is generally assumed that the major conformation of DNA in biological systems is B-DNA. An alternative conformation, termed Z-DNA, was recently described. Left-handed Z-DNA has been found to occur in fragments of natural DNA, supercoiling being a major factor to stabilize this conformation (for review see Refs. 1–4 and references therein). Immunological assays using antibodies to Z-DNA provide strong evidence for the presence of Z-fragments in chromosomes of two dipterian species (5, 6) and in the nuclei of mammalian cells (7, 8). The biological role of Z-DNA is not yet known. However, the consequences of the presence of Z-DNA fragments can be evaluated from the ability of cellular proteins to use Z-DNA as substrate. Proteins which specifically bind to Z-DNA have been described (9). Interactions between Z-DNA and several proteins, RNA polymerases (10, 11), histones (12, 13), methylases (14–16), nucleases (3, 15, 16), and DNA-glycosylase (17) have been studied.

Upon alkylation of DNA by chemical carcinogens such as N-methylnitrosourea or N-methyl-N-nitro-N-nitrosoguanidine, the main reaction products are 7-methylguanine, 3-methyladenine, O\textsuperscript{6}-methylguanine (O\textsuperscript{6}-MeGua\textsuperscript{*}), and the phosphotriesters (18). Lesions introduced in DNA by alkylating agents are repaired either by a DNA alkyltransferase or by the sequential action of a DNA glycosylase and an apurinic/apyrimidinic endonuclease (for review see Refs. 18 and 19). We have shown that the imidazole ring-opened form of 7-methylguanine was not excised by the specific DNA-glycosylase when this lesion was present in poly(dG-me\textsuperscript{6}dC) in Z-form (17). So far, the mutagenic and/or carcinogenic properties of alkylating agents are correlated with the persistence of O\textsuperscript{6}-methylguanine residues in DNA (18). Mammalian and bacterial cells contain a protein, the O\textsuperscript{6}-methylguanine-DNA methyltransferase, which repairs the O\textsuperscript{6}-alkylguanine by transferring the alkyl group to one of its own cysteine residues, restoring the guanine in DNA in a single step (for review see Ref. 19).

In this study, we measured the effects of the conversion of the B- to the Z-conformation of a polynucleotide containing O\textsuperscript{6}-methylguanine residues, on Escherichia coli O\textsuperscript{6}-MeGua\textsuperscript{*}-DNA methyltransferase activity. We report that O\textsuperscript{6}-methylguanine is very poorly repaired, if at all, when the alkylated poly(dG-me\textsuperscript{6}dC) is converted to the left-handed Z-form.

**EXPERIMENTAL PROCEDURES**

Preparation of Substrates Containing O\textsuperscript{6}-Methylguanine Residues—
Calf thymus DNA (Chassy Products, Paris), poly(dG-dC)-poly(dG-dC), or poly(dG-me\textsuperscript{6}dC)-poly(dG-me\textsuperscript{6}dC) (P-L Biochemicals) were diluted in 0.3 M Na cacodylate, 0.1 M perchloric acid buffer, pH 7.5, and alkylated with 1 mCi of N\textsuperscript{3H}-methyl-N-nitrosourea (2.9 Ci/ mmol; Amersham). The reaction was carried out at 37 °C for 4 h. Under these conditions, less than 0.5% of the total bases were modified. The specific activities of MNU-DNA, MNU-poly(dG-dC),


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*This work was supported by Institut National de la Sante et de la Recherche Medicale Grant U 140 and by Centre National de la Recherche Scientifique Grant LA 147. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ This work was supported by Institut National de la Sante et de la Recherche Medicale Grant U 140 and by Centre National de la Recherche Scientifique Grant LA 147. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
and MNU-poly(dG-m6dC) were 100, 500, and 1400 cpm/nmol, respectively. O6-methylguanine accounted for 6, 14, and 11% of the total radioactivity, respectively.

**O6-Methylguanine-DNA Methytransferase Assay—** The standard assay measured the disappearance of O6-methylguanine from [3H]MNU-treated DNA (30). The reaction mixture (100 µl), contained 50 nmol of [3H]MNU-DNA (0.1 pmol of O6-methylguanine) in 5 mM Hepes-KOH (pH 7.5), 5 mM dithiothreitol, 1 mM EDTA, 50 mM NaCl, and 0-0.5 units of O6-MeGua-DNA methyltransferase. Alternatively, 3 nmol of [3H]MNU-poly(dG-dC) or 2 nmol of [3H]MNU-poly(dG-m6dC) were used as substrates (~0.1 pmol of O6-MeGua). The reaction was carried out at 25 °C for 10 min. The mixture was then supplemented with authentic markers (7-methylguanine and O6-MeGua) and 0.1 M HCl (final concentration). The resulting mixture was heated for 30 min at 70 °C, cooled and centrifuged in Eppendorf microtubes, and neutralized with NaOH, and the products were separated by high pressure liquid chromatography. Alternatively, the hydrolysates were filtered through GF/C filters (Whatman), and the radioactivity bound to the filters reflected the methyltransferase activity. This assay was used during the course of the purification. However, each peak of activity was further measured by high pressure liquid chromatography because of some methyltransferase positive responses.

**Preparation of E. coli O6-Amethylguanine-DNA Methytransferase—** The protein was purified from E. coli BS 21 (adc thy his), a strain constitutive for the synthesis of the O6-MeGua-DNA methyltransferase (20). The cells were grown at 37 °C in LB broth to late exponential phase. Bacteria were centrifuged, washed, resuspended in lyso buffer (20 mM Hepes-KOH, pH 7.5, 5 mM dithiothreitol, 0.5 mM NaCl, EDTA), and stored at ~70 °C. The cells (5 g) were lysed using lysosome as already described (21). All subsequent steps were performed at 4 °C. The lysate was sonicated to reduce viscosity and centrifuged by centrifugation (25,000 X g). The supernatant was used as the source for the methyltransferase (Fig. 1). The enzyme was defined as the amount of protein needed to remove 1 pmol of O6-Methylguanine from MNU-treated calf thymus DNA at 25 °C in 10 min.

**Activity of O6-Methylguanine-DNA methyltransferase using as substrate MNU-poly(dG-dC).** The disappearance of O6-methylguanine from the substrate was measured at 25 °C for 10 min as described under “Experimental Procedures.” The units given in the abscissa were determined using MNU-DNA. Open symbols, assays performed in the absence of MgCl2; the substrate was preheated at 50 °C for 10 min (O—O) or not (Δ—Δ) previous to the O6-MeGua-DNA methyltransferase reaction. Closed symbols, assays performed in the presence of 4 mM MgCl2, the substrate being preheated at 50 °C for 10 min (●—●) or not (■—■) before the O6-MeGua-DNA methyltransferase reaction.
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FIG. 2. Activity of O6-methylguanine-DNA methyltransferase using as substrate MNU-poly(dG-meSdC) in B- or Z-form. The assay conditions and the symbols were as in Fig. 1. Inset, the ratio A295/A260 of a 100 mM solution of MNU-poly(dG-meSdC) in the O6-MeGua-DNA methyltransferase buffer was measured. The template was heated at 50 °C in the presence of MgCl2 and the absence of MgCl2 or heat treatment alone of MNU-poly(dG-meSdC) prior to incubation with O6-MeGua-DNA methyltransferase, neither modifies the A295/A260 ratio, the CD spectrum, nor the activity of the O6-MeGua-DNA methyltransferase. Incubation of MNU-poly(dG-meSdC) for 10 min at 25 °C (which are the assay conditions for O6-MeGua-DNA methyltransferase) does not induce significant conversion, if any, to the Z-form, as judged by the A295/A260 ratio or CD spectrum which remains unchanged (not shown). By addition of 4 mM MgCl2 and heat treatment for 10 min at 50 °C, the MNU-poly(dG-meSdC) adopts the Z-conformation, as judged by the increase of the A295/A260 ratio (Fig. 2, inset) and circular dichroism (Fig. 3). Fig. 2 shows that O6-MeGua residues are poorly repaired, if MNU-poly(dG-meSdC) is converted to the Z-form. The amount of O6-MeGua repaired in the left-handed polymer is at the threshold of the detection and is not modified by the addition of a large excess of protein (Fig. 2). The MNU-poly(dG-meSdC) in Z-conformation cannot be sedimented out of solution (data not shown) as described for Z*-poly(dG-dC) (25), suggesting the absence of intermolecular aggregation. Furthermore, the lack of activity of the O6-MeGua-DNA methyltransferase is not due to heat treatment in the presence of MgCl2, as MNU-poly(dG-dC) treated under the same conditions remains a good substrate (Fig. 1).

The kinetics of conversion of MNU-poly(dG-meSdC) to the Z-form in the presence of MgCl2 was measured at 37 °C. Fig. 4 (inset) shows that half of the transition is observed within 10 min, and it is completed after 30 min. Taking advantage of the very low, if any, conversion at 25 °C (not detectable within 10 min), the activity of O6-MeGua-DNA methyltransferase can be measured using as substrate the MNU modified polynucleotide containing increasing amount of Z-form. Fig. 4 shows that the kinetics of inactivation of the O6-MeGua-DNA methyltransferase is well correlated with the kinetics of conversion from the B-form to the Z-form. These results again suggest that O6-MeGua is not repaired by the O6-MeGua-DNA methyltransferase when present in polymer in the left-handed Z-conformation. The lack of activity is not due to an irreversible inactivation of the enzyme, as O6-MeGua-DNA methyltransferase incubated in the presence of a substrate in Z-form remains able to repair subsequently added substrate in B-form (not shown).

Drugs that intercalate in DNA, such as ethidium bromide, induce the transition from the Z-form to the B-form (26, 27).

MeGua-DNA methyltransferase (Fig. 2). It appears that there is a slight difference in the maximum level of activity that O6-MeGua-DNA methyltransferase can reach with MNU-poly(dG-dC) and poly(dG-meSdC) in the B-form (Figs. 1 and 2). This difference seems to be due to intrinsic properties of the polynucleotides rather than to the presence of a fraction of the MNU-poly(dG-meSdC) in the Z-form (Figs. 2 and 3). Figs. 2 and 3 show that addition of 4 mM MgCl2 or heat treatment alone of MNU-poly(dG-meSdC) prior to incubation with O6-MeGua-DNA methyltransferase, neither modifies the A295/A260 ratio, the CD spectrum, nor the activity of the O6-MeGua-DNA methyltransferase. Incubation of MNU-poly(dG-meSdC) for 10 min at 25 °C (which are the assay conditions for O6-MeGua-DNA methyltransferase) does not induce significant conversion, if any, to the Z-form, as judged by the A295/A260 ratio or CD spectrum which remains unchanged (not shown). By addition of 4 mM MgCl2 and heat treatment for 10 min at 50 °C, the MNU-poly(dG-meSdC) adopts the Z-conformation, as judged by the increase of the A295/A260 ratio (Fig. 2, inset) and circular dichroism (Fig. 3). Fig. 2 shows that O6-MeGua residues are poorly repaired, if MNU-poly(dG-meSdC) is converted to the Z-form. The amount of O6-MeGua repaired in the left-handed polymer is at the threshold of the detection and is not modified by the addition of a large excess of protein (Fig. 2). The MNU-poly(dG-meSdC) in Z-conformation cannot be sedimented out of solution (data not shown) as described for Z*-poly(dG-dC) (25), suggesting the absence of intermolecular aggregation. Furthermore, the lack of activity of the O6-MeGua-DNA methyltransferase is not due to heat treatment in the presence of MgCl2, as MNU-poly(dG-dC) treated under the same conditions remains a good substrate (Fig. 1).

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Drugs that intercalate in DNA, such as ethidium bromide, induce the transition from the Z-form to the B-form (26, 27).

FIG. 3. Circular dichroism spectra of MNU-poly(dG-meSdC). The MNU-poly(dG-meSdC) was diluted to a final concentration of 100 μM in the O6-MeGua-DNA methyltransferase buffer. Panels A and B show the CD spectra in absence of MgCl2; the polynucleotide was preheated for 10 min at 50 °C (B) or not (A). Panels C and D show the CD spectra in the presence of 4 mM MgCl2; the polynucleotide was preheated for 10 min at 50 °C (D) or not (C).
Fig. 5 shows that when MNU-poly(dG-me6dC) is in the Z-form, the O6-MeGua is not removed from the polynucleotide, as already shown above. Upon addition of ethidium bromide, the complex ethidium bromide-MNU-poly(dG-me6dC) becomes substrate for the O6-MeGua-DNA methyltransferase in a cooperative manner. The transition was assessed using antibodies to Z-DNA, and the sigmoidal curve observed is identical to that obtained for O6-MeGua-DNA methyltransferase activity (Fig. 5). The amount of ethidium bromide required to induce 50% transition is 0.1 ethidium bromide molecule/nucleotide. When the amount of ethidium bromide is higher than 1 molecule/nucleotide, the O6-MeGua-DNA methyltransferase is inhibited, the template being either MNU-poly(dG-me6dC) (not shown). Furthermore, reversion from the Z- to the B-form can be induced by addition of EDTA (25). The addition of an excess of EDTA, relative to MgCl2, to MNU-poly(dG-me6dC) in Z-form, completely restores the ability of the polymer to be a substrate for O6-MeGua-DNA methyltransferase (not shown). These two last results show a high correlation between the conditions which allow the Z-to-B transition and the recovery of the O6-MeGua-DNA methyltransferase activity.

**DISCUSSION**

The ability of DNA repair enzymes and/or proteins to maintain the genome's integrity is of major importance for the cell. So far, the initial step of the repair of DNA methylated by chemical carcinogens depends upon DNA-glycosylases and DNA-methyltransferases (18, 19, 21). In vitro experiments have shown that the imidazole ring-opened form of 1-methylguanine is not repaired by the specific DNA-glycosylase when this lesion is in Z-DNA (17). We show that this holds true for the repair of O6-MeGua when in Z-DNA. This lesion is repaired by the direct transfer of the methyl group from the modified base to one of the cysteine residues of O6-MeGua-DNA methyltransferase. Therefore, two distinct classes of repair enzymes are not able to repair lesions when present in Z-DNA. Similarly, the HhaI methylase or restriction endonuclease (15, 16) are not able to act on DNA in the Z-form. These results demonstrate that neither the DNA lesion itself (Ref. 17 and this work) nor the primary base sequence (15, 16) are the only basis for the formation of proper substrate for these proteins. The structure of the helix plays also an important role, either on the recognition of the lesion or on the reaction. In order to increase the biological relevance of the effect described in this paper, it would be important to show that it remains true in natural DNA, where the B→Z transition would be driven by supercoiling.

Therefore, the lack of repair of O6-MeGua residues in Z-DNA may result in the persistence of such lesions in the cellular genome. As the mutagenic and carcinogenic potency of alkylating agents is reasonably associated with the ability to produce O6-MeGua and to the lack of repair of this lesion (28), one can propose that the formation of O6-methylguanine in DNA fragments which adopt the Z-conformation will increase the mutagenic effect of O6-MeGua at these sites. The physiological methylation of the cytosine residues in eukaryotic systems (29) could provide repeating sequences which could easily adopt the Z-conformation. These DNA fragments could display an unexpected high mutation rate after treatment with chemical carcinogens. This possibility was proposed to occur in N-acetoxy-N-2-acetylaminofluorene-induced mutation, as it is observed at a limited number of sites (30). The spontaneous mutagenesis might be also greatly increased if the enzymes which control the repair of mismatched bases (31) are similarly inhibited. Therefore mutational hot spots could be a consequence of Z-DNA conformation as previously suggested for quasi palindromic sequences (32). The occurrence of Z-DNA fragments in the genome could provide sequences with a high mutation rate, whereas the major part of the genome (in B-form) will display a low mutation rate. The hypothesis would imply an evolutionary function for Z-DNA.

**Acknowledgments**—We thank Drs. Leng, Malfoy, and Malinge, Centre de Biophysique Moléculaire, Orléans, France, for the gift of antibodies and circular dichroism measurements. We are grateful to C. Lagravère for her excellent technical assistance.

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