Inhibition of Human O6-Methylguanine-DNA Methyltransferase by 5-Methylcytosine

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Abstract

The ability of cloned human O6-methylguanine-DNA methyltransferase to repair a methylated guanine in a CpG-containing sequence, i.e., island, was studied by using a synthetic double-stranded 20-mer oligonucleotide from codon 248 of the p53 gene and another designed sequence. The double-stranded oligonucleotides incorporating 5-methylcytosine (5mC) and O6-methylguanine (O6mG) in various combinations in a CpG site were 5' labeled with 32P and incubated with recombinant O6-methylguanine-DNA methyltransferase. The rate constant for O6-methylguanine-DNA methyltransferase repair of O6mG in this oligomer was always higher with the substrate which contained only the O6mG, as compared to the oligomer that included a 5mC adjacent in the S'-position to the methylated guanine. The reduction in substrate activity ranged from 75% (modified p53 sequence) to 100% (in the designed oligomer). A 5mC opposite the O6mG reduced the rate slightly. These results suggest that O6-methylation of the guanine moiety at CpG islands may not be efficiently repaired when normal 5mC is present and this may contribute significantly to an increase in mutagenesis of p53 and like molecules.

Introduction

MGMT3 (EC 2.1.1.63) is a protein that specifically transfers the alkyl group on the exocyclic oxygen of guanine residues to a protein (1, 2). Alkylation of guanine on this oxygen has been shown to produce mispairing during DNA replication leading to GC-AT transitions (3). The persistence of this lesion contributes to mutagenesis in bacterial test systems and is strongly implicated in the tumorigenic potential of certain alkylating agents (4, 5). While differences in molecular weight, rate constants, and substrate specificities exist, MGMT is present in a wide range of organisms from bacteria to humans (6). MGMT from all species studied to date exhibit the same basic reaction mechanism for removal of the alkyl group from the O6-position. The protein transfers the alkyl group from the alkylated guanine to an internal cysteine residue, thus restoring the integrity of the DNA, while at the same time, the protein is inactivated. Mammalian MGMT is very efficient in removing methyl groups from methylated guanine in DNA, but longer chain alkyl groups are poorer substrates; the bacterial protein is somewhat more effective at removal of longer chain alkyl groups (6, 7).

Several factors are known to alter the rate of demethylation of O6-methylguanine in DNA by MGMT. Among these are temperature, salt concentration, presence of metal ions, and the extent of depurination in the DNA substrate (8-10). In addition, Georgiadis et al. (11) reported that moving the O6-methyl from the first to the second guanine in codon 12 of the ras oncogene can significantly change the repair rate. Therefore, sequence and structural modifications of the DNA substrate affect the extent of repair by MGMT. Many genes are regulated by methylation of the cytosine moiety of CpG dinucleotide sites at the 5-position (12), but nothing is known about how this nucleotide affects MGMT activity. The p53 gene was chosen for study because it is commonly found to be mutated in a number of different cancers (13). We used an 18-mer which contained codon 248. This codon (CGG) has a CpG site which is known to be mutated in some cancers (13-15).

Materials and Methods

Materials. All chemicals which were reagent grade or better were obtained from Sigma Chemical or Fisher Scientific Companies unless stated otherwise. The p53 oligonucleotides were purchased from Macromolecular Resources, Fort Collins, CO. They were deblocked in ammonium hydroxide (p53wt, p53Cmth, p53Comp) or 1, 8-diazaibicyclo[5.4.0]undec-7-ene (p53Cmth, p53Cmth, p53Cmth) as recommended by the manufacturer. Deblocked oligonucleotides were purified over a Nensorb Prep column and checked for purity by ion-exchange chromatography. The designed 20-mer oligonucleotides were synthesized by using a Cyclone DNA Synthesizer (Milligen). After synthesis, the oligodeoxynucleotides were purified initially by reverse-phase HPLC and then by ion-exchange HPLC. After the ion-exchange step, a buffer exchange was performed by ultrafiltration and the concentration of DNA was assayed by UV absorbance. The human MGMT used in this study was obtained from an MGMT cDNA that had been cloned into an Escherichia coli expression vector system as previously described (10). Briefly, human MGMT cDNA was cloned into a PET high-expression vector (Novagen Inc, Madison, WI) and transfected into E. coli BL21 (DE-3). One-liter cultures grown in LB medium with glucose and ampicillin were induced with isopropyl-1-thiogalactopyranoside. The bacteria were disrupted by sonication and the protein was purified by ammonium sulfate precipitation, dialysis, low-pressure chromatography on DEAE-Sephrose chromatography on Mono S (Pharmacia, Inc.). Protein activity was checked by using N-methyl-N-nitrosourea-treated calf thymus DNA as previously described (10).

Preparation of Double-Stranded Oligomeric Substrate. Purified oligonucleotides were 32P-labeled on the 5' end by using T4 polynucleotide kinase (USB Corp., Cleveland, OH) essentially as described, except that 15 pmol of substrate and 20 pmol of y-32PATP (7000 Ci/mmol; Amersham, Inc., Irvine, CA) were incubated with 6-7.5 units of enzyme. Excess ATP was removed from the labeled oligonucleotide by passing the reaction mix over a NAPtrap resin column (Stratagene, Inc., La Jolla, CA). The labeled, purified products were annealed to a slight excess of oligomeric complementary strand, and the double-stranded material was desalted through a NAP-10 column (Pharmacia, Inc.). The eluted double-stranded DNA was lyophilized to a volume of about 10 μl.

MGMT Reaction. The MGMT reaction consisted of 9.2 pmol of labeled double-stranded oligonucleotide and 5.8 pmol MGMT in 50 mM Tris (pH 7.8), 4.5 mM dithiothreitol, 0.8 mM EDTA, 5% glycerol, 200 μg/ml bovine serum albumin in a reaction volume of 250 μl. A 20-μl aliquot which was removed before addition of MGMT was used as time 0. After addition of MGMT, 20-μl aliquots were removed at 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, and 30.0 min, were diluted in 500 μl of Buffer A (0.35 mM NaCl in 0.01 mM NaOH, pH 12) and frozen until HPLC analysis.

HPLC Analysis. Samples were analyzed by ion-exchange HPLC with the use of a Waters two-pump system with a programmable gradient controller (Waters/Millipore, Milford, MA). A HEMA Bio-Q column (4.6 mm × 50 mm) obtained from Alltech Instruments (Deerfield, IL) was used in this...
analyses. Separation of the methylated and demethylated oligomers was achieved by a 1%/min gradient from 100% Buffer A to 30% Buffer B (1.2 mM NaCl in 0.01 M NaOH, pH 12) over 30 min, at a flow rate of 1.0 ml/min. Fractions were collected and the radioactivity was quantitated by liquid scintillation spectrometry.

Calculations. The rate constants were calculated as described previously (7, 10) from the second order rate equation,

$$k = \frac{1}{B_0 - A_0} \ln \left( \frac{B_0 - B_t}{A_0 - A_t} \right)$$

where $k$ is the rate constant; $B_0$ and $A_0$ the concentration of reactants at time 0, and $B_t$ and $A_t$ are the concentration of reactants at time $t$.

Results and Discussion

The oligonucleotides used in this study are presented in Table 1. The reaction of MGMT with the 20-mer double-stranded O6-methylguanine-containing oligonucleotide was quite rapid as can be seen in Fig. 1. Repair of the double-stranded oligomers, GMTH:COMP and GMTH:MCOMP, was nearly complete between 2 and 5 min. The calculated rate constant for repair of the O6-methylguanine-containing oligonucleotide, GMTH:COMP, was $3.6 \times 10^7$ M$^{-1}$ s$^{-1}$ (Table 2). When 5-methylcytosine was placed opposite the O6-methylguanine in the complementary strand, i.e., GMTH:MCOMP, $k$ was reduced somewhat to $2.1 \times 10^7$ M$^{-1}$ s$^{-1}$. The incorporation of a 5-methylcytosine 5’ to a O6-methylguanine moiety in the double-stranded oligomer resulted in complete loss of substrate activity for human MGMT (Fig. 1). The complete loss of substrate activity was noted with both DMTH:COMP and DMTH:MCOMP (Fig. 1). Since no detectable product was formed in these reactions during the time period examined, no rate constant could be calculated. The incubation has been conducted up to 2 h at 37°C without the appearance of any detectable demethylated product (data not shown).

Since the result of the first experiment was so dramatic, further investigations were performed by using a sequence from the p53 gene encompassing codon 248 which contains a CpG site. Incubation of the 18-base pair sequence containing different methylation patterns also showed a marked reduction in the rate of repair by MGMT. Fig. 2 shows the results of the reaction of MGMT with methylated p53. Although not as dramatic as the previous experiment, the rate of repair of the O6-methylguanine in the codon 248 CpG site was reduced by 75% when 5-methylcytosine was the 5’-nucleotide. The $k$ values were determined at 0°C to allow more accurate estimation of the rate constants. The same reduction in repair rate was seen at 10°C and 25°C, although the overall rate was similar to the GMTH oligonucleotide (data not shown). A summary of the kinetic constants is presented in Table 2.

The rate constants for the repair of the double-stranded O6-methylguanine-containing oligomers were about an order of magnitude less than that which we have previously reported (10). Using methylated calf thymus DNA, we had estimated the $k_t$ as $3.3 \times 10^8$ M$^{-1}$ s$^{-1}$ at 25°C (10). It is quite likely that the substrate efficacy for MGMT is markedly dependent upon the length of the oligomer and the extent of alteration of the bases. In our previous study, a greater rate of repair
by MGMT was observed when “native” methylated DNA was used as substrate compared to partially depurinated methylated DNA (10).

Graves et al. (7) have reported k values of $2.5 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ with self-complementary 12-mers as substrates for the E. coli Ada protein. This value is almost identical to the k reported in the present study. Georgiadis et al. (11) have examined the repair of O6-methylguanine in 15-mers that contained codon 12 (GGG) of the H-ras oncogene. The investigators noted a rate of repair which was 18 times slower for the GmGA sequence versus the mGGA sequence. Their reported k values were in the range of $10^4$ to $10^5 \text{M}^{-1} \text{s}^{-1}$. Although the reason for the large difference in repair rate from earlier reported values is not clear, there is agreement that the position of the methyl group can be a critical factor in determining the rate of MGMT activity. It is also consistent with our observation that sequence is an important determinant of the rate of repair.

Although the general mechanism of MGMT action is known, the details of how the methyl group is transferred from the purine to the protein remain elusive. It has long been known that the methyl moiety is transferred to a cysteine contained in a Pro-Cys-His-Arg-Val sequence which is present near the NH2-terminus MGMT (or Ada). This is transferred to a cysteine contained in a Pro-Cys-His-Arg-Val sequence which is present near the NH2-terminus MGMT (or Ada). This sequence is conserved in the repair protein in the human, rat, yeast, E. coli, Salmonella typhimurium, and Bacillus subtilis. (16) Spratt and de los Santos (17) have proposed a tentative mechanism for MGMT action wherein the methylguanine is held in place by hydrogen bonding to the protein. The methyl group is displaced from the exocyclic oxygen through a nucleophilic attack by the cysteine sulfur. A proton must be donated to the guanine oxygen by another amino acid in or near the active site. Conceivably, the presence of a 5-methylcytosine adjacent to the O6-methylguanine either prevents the proper orientation within the active site or blocks the donation of the proton to the guanine. Another possibility is that interaction of 5-methylcytosine and O6-methylguanine changes the local conformation of the DNA which impedes recognition and/or binding by MGMT. Model systems such as the one presented here could help in the deciphering of the details of this reaction.

Poor repair of DNA damage at CpG sequences can have a potential impact on the occurrence of mutation and carcinogenesis. This lack of repair in the p53 tumor suppressor gene may be responsible at least in part for its activation to an oncogene. p53 is frequently found mutated in colorectal, lung, and soft tissue cancers (13–15). Several of the previously reported mutations involve G to T transversions of guanines located in CpG islands. In addition, mutations at several sites, including codon 248, significantly altered the normal effect of p53 on transcription (18). At least 35 CpG dinucleotide sequences occur in the coding region of the human p53 cDNA (as published in GenBank). However, the methylation status of the cytosine residues in these CpG sequences is not known. It is possible that one of the reasons for the higher mutation frequency at these sites is the lack of repair of O6-methylguanine formed by endogenous methylating agents or by exogenously-administered prodrugs that are activated to methylating agents.

Given the important role that methylation at the 5 position of cytosine plays in the regulation of gene expression (11, 19), it is intriguing that an endogenous repair system could be significantly inhibited by normal methylation of CpG sites. This inhibition may partially explain the observation that CpG dinucleotides are under-represented in the vertebrate genome while CpG sequences occur at a greater than expected level (20). In addition, recent work has shown that certain proteins exist which specifically bind to methylated CpG islands (21, 22) and that this interaction may be a necessary component of a regulatory mechanism. Unrepaired O6-methylguanine in the CpG sequence might prevent the normal binding of these factors. At present, insufficient information exists on the actual protein-DNA interaction to allow a more detailed description of the 5-methylocytosine-mediated inhibition. A better understanding of the mechanism of MGMT repair is needed before this inhibition can be fully explained.

References


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