Redesigning the Substrate Specificity of Human O^6-Alkylguanine-DNA Alkyltransferase. Mutants with Enhanced Repair of O^4-Methylthymine†

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ABSTRACT: Human O^6-alkylguanine-DNA alkyltransferase (MGMT) repairs potentially cytotoxic and mutagenic alkylation damage at the O^6-position of guanine and the O^4-position of thymine in DNA. We have used random sequence mutagenesis and functional complementation to obtain human MGMT mutants that are resistant to the MGMT inhibitor, O^6-benzylguanine [Encell, L. P., Coates, M. M., and Loeb, L. A. (1998) *Cancer Res. 58*, 1013−1020]. Here we describe screening of O^6-benzylguanine-resistant mutants for altered substrate specificity, i.e., for an increased level of utilization of O^4-methylthymine (m^4T) relative to that of O^6-methylguanine (m^6G). One mutant identified by the screen, 56-8, containing eight substitutions near the active site (C150Y, S152R, A154S, V155G, N157T, V164M, E166Q, and A170T), was purified and characterized kinetically. The second-order rate constant for repair of m^4T by the mutant was up to 11.5-fold greater than that of WT MGMT, and the relative m^4T specificity, $k(m^4T)/k(m^6G)$, was as much as 75-fold greater. In competition experiments with both substrates present, the mutant was 277-fold more sensitive to inhibition by m^4T than WT MGMT. This mutant, and others like it, could help elucidate the complex relationship between adduction at specific sites in DNA and the cytotoxicity and mutagenicity of alkylating agents.

Alkylating agents produce a multitude of DNA alterations that are mutagenic in the absence of repair (*1*, *2*). O^6-Methylguanine (m^6G)[1] is the most abundant mutagenic lesion induced by simple methylating agents (*3*). O^4-Methylthymine (m^4T) is less frequently formed, but is more mutagenic in both prokaryotic and eukaryotic cells (*4*−*9*). m^6G and m^4T residues in DNA induce G → A and T → C mutations, respectively, and a role for both adducts in initiating carcinogenesis has been proposed on the basis of their persistence in animals that develop tumors following treatment with alkylating agents (*10*−*13*). Because the two adducts exhibit similar mispairing proficiencies in vitro (*14*), the greater mutagenicity of m^4T has been ascribed to lack of repair, and this is in accord with the longer persistence of m^4T adducts in the DNA of repair-proficient cells and animals (*15*, *16*). In humans, both adducts are repaired by O^6-alkylguanine-DNA alkyltransferase (MGMT, EC 2.1.1.63). MGMT, also called AGT (*17*, *18*), repairs these adducts by a direct stoichiometric transfer of the alkyl group to the repair protein (*19*). A unique cysteine residue accepts the alkyl group, forming a stable, thioether linkage (*20*, *21*). MGMT is frequently classified as a suicide protein because the cysteine sulfhydryl moiety is not regenerated, resulting in irreversible inactivation. Wild-type (WT) MGMT repairs m^6G more efficiently than m^4T, although the magnitude of this substrate preference has varied in recent studies (*21*−*23*).

We have previously employed random sequence mutagenesis to create a large library (>10^6 clones) of MGMTs containing mutations near the active site cysteine (*17*, *24*). The library was expressed in alkyltransferase-deficient *Escherichia coli*, and cells were treated with the methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) together with the competitive MGMT inhibitor O^6-benzylguanine (BG). In this way, we selected mutants that are highly resistant to BG yet maintain the ability to protect against killing by MNNG.

The mechanism(s) by which MGMT protects cells from damage by alkylating agents has been difficult to establish because these agents produce numerous alterations in DNA, any of which could be rate-limiting for survival or mutagenesis. MGMT's primary role may be in limiting mutations because its two substrates, m^6G and m^4T, have mispairing potential. However, MGMT also reduces cytotoxicity (*25*, *26*). The presumptive lethality of m^6G and/or m^4T is believed to be due to strand scissions induced by repeated, futile attempts of the mismatch repair system to remove the adducts (*27*). Neither m^6G nor m^4T appears to be a strong block to replication, since each is readily copied past by purified DNA polymerases (*28*, *29*). 3-Methyladenine is probably the adduct most responsible for cytotoxicity, apparently due to its potency in blocking DNA polymerases during replication (*30*, *31*); however, 3-methyladenine is not a substrate for MGMT.

Creating mutant MGMTs with altered specificities against m^4T and m^6G could provide us with powerful probes for understanding the relative contribution by each of these DNA

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[1] Abbreviations: m^6G, O^6-methylguanine; m^4T, O^4-methylthymine; MGMT, human O^6-alkylguanine-DNA alkyltransferase; WT, wild-type; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; BG, O^6-benzylguanine; PAGE, polyacrylamide gel electrophoresis; MNU, *N*-methyl-*N*-nitrosourea.

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adducts to the biological effects of alkylating agents. In the absence of agents that specifically alkylate DNA at the O^4-position of thymine, it is difficult to directly select mutants that exhibit enhanced repair of m^4T. However, we reasoned that among our mutants that are resistant to BG there would be some with altered substrate specificity, because the BG-resistant phenotype most likely results from a structural alteration near the active site that prevents entry of the bulky BG group (*18*). Indeed, by screening BG-resistant variants, we were able to identify mutants that preferentially repaired m^4T. One such mutant, with eight amino acid substitutions near the active site, is analyzed here in detail.

EXPERIMENTAL PROCEDURES

Materials. E. coli GWR111 (ada ogt; *32*) was a gift from L. Samson. *E. coli* NM522 (Stratagene) was used for cloning. *E. coli* BL21(DE3) (Novagen) was used for expression and purification of alkyltransferases. 1× YT medium (*33*) with the indicated antibiotics was used for bacterial growth unless otherwise noted. pUC118-MGMT vectors were constructed as described previously (*17, 34*) from pKT100 (*35*), which was generously provided by S. Mitra. Vector pET28a and the His tag protein purification kit were from Novagen. O^6-Methyldeoxyguanosine and O^4-methyldeoxythymidine phosphoramidites were from Glen Research, and oligonucleotides were from Midland Certified Reagent Co. [γ-^{32}P]-ATP was from Amersham. All enzymes were from New England Biolabs, and all other reagents were from Sigma unless otherwise noted.

Oligonucleotide Substrates. Synthetic oligonucleotides, obtained from Midland, were synthesized by using the modified phosphoramidites and purified by PAGE The sequences of the 23-mer oligonucleotides were as follows: m^6G-1, 5′-GAACTG*CAGCTCCGTGCTGGCCC-3′; m^4T-1, 5′-GAACT*GCAGCTCCGTGCTGGCCC-3′; and m^4T-2, 5′-GAACGGCAGCT*GCAGGCTGGCCC-3′. G* and T* represent m^6G and m^4T, respectively, and the underlining indicates sequences that are substrates for cleavage by *Pst*I in the corresponding, unmethylated DNA duplexes. Methylated oligonucleotides (20 pmol) were 5′-labeled with [γ-^{32}P]-ATP (30 pmol) and separated from unincorporated ATP by using G-50 fine NICK spin columns (Pharmacia). Each labeled oligonucleotide (10 pmol) was annealed to a complementary oligonucleotide of the same length to form a duplex containing cytosine opposite m^6G or adenine opposite m^4T. Annealing was carried out in the presence of 15 pmol of complementary oligomer (to ensure complete hybridization of the labeled oligomer) by heating to 95 °C and then slowly cooling to room temperature.

Preparation of Bacterial Lysates. GWR111 cells harboring WT or mutant MGMT in pUC118 vectors were grown at 37 °C in 5 mL of 1× YT medium supplemented with 50 μg/mL carbenicillin and 25 μg/mL chloramphenicol. When the A_{600} reached 0.7, IPTG was added to a final concentration of 0.5 mM and the cultures were grown for an additional 3 h. Cells were harvested at 4 °C, resuspended in 250 μL of alkyltransferase buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 5 mM DTT] containing lysozyme (0.4 mg/mL), and frozen at −80 °C for approximately 12 h. Cells were lysed by slowly thawing on ice for a period of 2 h, and the lysates were centrifuged (27000*g*) at 4 °C for 30 min. Supernatants were removed; glycerol was added to a final concentration of 10%, and aliquots were stored at −80 °C. Protein concentrations were determined by the Bradford dye-binding procedure with BSA as a standard (*36*).

Purification of Alkyltransferases. To construct vectors for overexpression of WT and mutant MGMTs, the appropriate cDNAs were subcloned from the pUC118-based vectors into pET28a by using PCR to amplify the insert and to introduce unique restriction sites near the ends for cloning. The PCR primers were 5′-GAT GCC CAT ATG GAC AAG GAT TGT G-3′ (forward) and 5′-CCG CTA AAG CTT CAT ACT CAG TTT CGG CCA G-3′ (reverse). Underlining indicates *Nde*I and *Hin*dIII restriction sites, respectively. The PCR products were digested with these restriction enzymes, gel-purified, and ligated into pET28a at the appropriate site. The resulting pET28a-based vectors encoded WT or mutant 56-8 MGMT fused at the N-terminus to 20 amino acids, including a hexahistidine sequence. Vectors were transformed into electrocompetent BL21(DE3) *E. coli* cells, and cells were grown at 37 °C in 500 mL of 2× YT medium supplemented with 50 μg/mL kanamycin. When the A_{600} reached 0.4, IPTG was added to a final concentration of 0.5 mM and the cells were grown for an additional 5 h. Cultures were harvested at 4 °C, and the pellets were resuspended in 30 mL of 1× binding buffer [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, and 5 mM imidazole] containing lysozyme (0.4 μg/mL) and frozen at −80 °C for 12 h. Cells were lysed by slowly thawing on ice over a period of 5 h, and the lysates were centrifuged (27000*g*) at 4 °C for 30 min. The hexahistidine-tagged MGMT fusion proteins were then purified at 4 °C via a single-step metal chelation chromatographic procedure by using Ni^{2+} affinity resin and the appropriate buffers for washing and eluting, according to the supplier. The supernatant was applied to a charged 10 mL His bind column (1 cm × 10 cm), and the column was extensively washed and then eluted at a flow rate of 20 mL/h; 1 mL fractions were collected. Nearly all of the recovered MGMT eluted in the first 5 mL, as determined by analysis of the fractions by 12% PAGE. Fractions containing MGMT were combined (2 mL) and dialyzed against 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 200 mM NaCl, and 10% glycerol for 16 h and then against the same buffer containing 1 mM DTT for an additional 8 h. The total amount of protein was again determined by the Bradford procedure, and the MGMT concentration was calculated by estimating the purity from Coomassie Blue-stained gels. Aliquots were stored at −80 °C.

DNA Repair Assay. To measure the extent of repair of m^6G and m^4T, we utilized ^{32}P-labeled, duplex oligonucleotide substrates containing the adducts within unique *Pst*I restriction sites. Following incubation with MGMT, the substrate was incubated with *Pst*I and the products were analyzed by PAGE. In this assay, unrepaired substrate is not cleaved by *Pst*I and migrates as a 23-mer; substrate that is repaired migrates as an 8-mer. Repair was visualized by autoradiography and quantitated by phosphor image analysis (Molecular Dynamics) of the resolved bands. This methodology has been used to measure alkyltransferase activity in vitro (*22, 34, 37, 38*).

Repair in Crude Lysates. For screening mutants, crude lysates of bacteria expressing WT or mutant MGMTs were incubated with radiolabeled oligonucleotides containing m^6G or m^4T in alkyltransferase buffer for 30 min at 37 °C in a

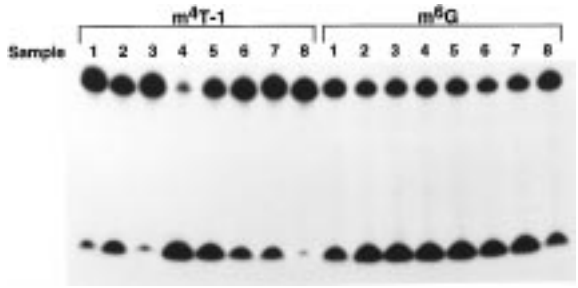

FIGURE 1: Repair of m^4T and m^6G by lysates of *E. coli* overexpressing WT or mutant MGMTs. Lysates were incubated for 30 min at 37 °C with ^{32}P-labeled duplex oligonucleotide substrates containing a single m^4T (eight lanes at left) or m^6G (eight lanes at right). Following digestion with *Pst*I, samples were electrophoresed on a 20% polyacrylamide gel. The lower bands in the autoradiogram are 8-mers produced by *Pst*I cleavage of the substrates after removal of the methyl group by MGMT. There was no evidence of a lower band in control reactions without alkyltransferase (data not shown). Samples were (1) WT MGMT, (2) V139F, (3) V139F/P140R/L142M, (4) mutant 56-8, (5) C150Y/A154G/Y158F/L162P/K165R, (6) S152T/G160L/E166D/A170I, (7) S152N/Y158H/L162H/W167L, and (8) G156A/G160R.

volume of 100 μL. The reactions were stopped by the addition of an equal volume of phenol. Following extraction with phenol, and extraction with equal parts phenol/chloroform−isobutanol (24/1) and chloroform/isobutanol (24/1), the oligonucleotides were precipitated by centrifugation from ethanol/300 mM sodium acetate (pH 5.2). The precipitates were washed with 70% ethanol and redissolved in 25 μL of H_2O. DNA (2 μL) was then digested with *Pst*I (0.5 unit) in a volume of 20 μL for 1 h at 37 °C. Radioactivity was determined by scintillation counting so that equal amounts could be loaded and resolved by 20% PAGE. Phosphor imaging was used to quantitate repair.

Repair by Purified Proteins. Purified WT or mutant MGMT (56-8) was incubated with labeled oligonucleotides containing m^6G or m^4T as described above, and reactions were stopped at various times for kinetic analysis. Rate constants were calculated from the following second-order rate equation (*39*)

$$kt = (1/B_0 - A_0) \ln[(B_0 - x)A_0/(A_0 - x)B_0]$$

where k is the rate constant, B_0 and A_0 are the concentrations of reactants at time zero, and $B_0 - x$ and $A_0 - x$ are the concentration of the reactants at time t. A plot of $\ln[(B_0 - x)A_0/(A_0 - x)B_0]$ as a function of time should be linear, and k can be calculated from the slope of the line $k(B_0 - A_0)$.

RESULTS

Repair of m^6G and m^4T by Bacterial Crude Lysates. The ability of WT and 14 mutant MGMTs to repair m^6G and m^4T was examined by overexpressing the proteins in alkyltransferase-deficient *E. coli* and incubating lysates with the m^6G and m^4T-1 substrates. Lysates (25 μg of total protein) were incubated with 0.2 pmol of oligonucleotide substrate in a total volume of 100 μL for 30 min at 37 °C, and the oligonucleotides were then digested with *Pst*I, and examined by 20% PAGE. Figure 1 shows results for WT MGMT (sample 1) and seven of the mutants (samples 2−8). Two of the mutants were created in an earlier library (*34*) and were included in the screen because they are known to provide enhanced protection against MNNG (V139F; sample 2) or resistance to BG (V139F/P140R/L142M; sample 3) when expressed in *E. coli* (*34*, *40*). As indicated by the gel, WT MGMT repaired m^6G more efficiently than m^4T-1. However, several mutants repaired m^4T-1 more efficiently than WT MGMT, particularly mutant 56-8 (C150Y, S152R, A154S, V155G, N157T, V164M, E166Q, and A170T; sample 4). Whereas WT MGMT repaired less than 10% of the m^4T substrate (lane 1), mutant 56-8 repaired more than 90% (lane 4); in contrast, there was no detectable difference in the levels of repair of m^6G. It should be noted that some of the mutants repaired m^4T-1 less effectively than WT MGMT (samples 3 and 8). On the basis of a series of screening assays such as those presented in Figure 1, we chose to purify mutant 56-8 for a more detailed characterization.

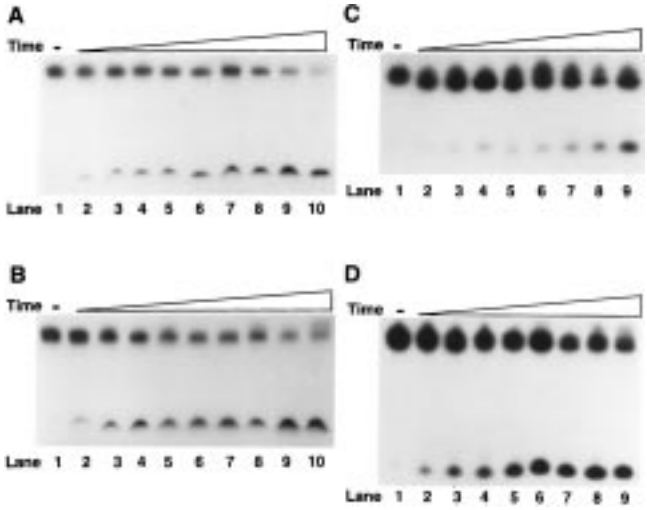

FIGURE 2: Time courses of m^4T-1 and m^6G repair by purified WT and mutant 56-8 MGMT. MGMT (0.10 pmol) was incubated with labeled m^4T-1 or m^6G substrates (0.13 pmol) for increasing periods of time at 37 °C. Following digestion with *Pst*I, samples were electrophoresed on a 20% polyacrylamide gel. The lower band in the autoradiograms represents repair of the methyl adduct. (A) Mutant 56-8 acting on m^4T-1: (1) no alkyltransferase control and repair for (2) 10 s, (3) 20 s, (4) 50 s, (5) 1.3 min, (6) 2 min, (7) 2.5 min, (8) 3 min, (9) 6 min, and (10) 10 min. (B) Mutant 56-8 acting on m^6G: (1) no alkyltransferase control and repair for (2) 20 s, (3) 50 s, (4) 1 min, (5) 1.3 min, (6) 2 min, (7) 2.5 min, (8) 3 min, (9) 6 min, and (10) 10 min. (C) WT MGMT acting on m^4T-1: (1) no alkyltransferase control and repair for (2) 20 s, (3) 45 s, (4) 1 min, (5) 2 min, (6) 3 min, (7) 5.3 min, (8) 10 min, and (9) 20 min. (D) WT MGMT acting on m^6G: (1) no alkyltransferase control and repair for (2) 10 s, (3) 22 s, (4) 33 s, (5) 45 s, (6) 56 s, (7) 1.5 min, (8) 2 min, and (9) 4 min.

Cloning and Overexpression of Alkyltransferases. WT MGMT and mutant 56-8 were cloned into pET28a and overexpressed in BL21(DE3), and the hexahistidine-tagged fusion proteins were purified by one-step Ni^{2+} affinity chromatography. The purity was estimated to be >90% by visual inspection of Coomassie Blue-stained gels.

Kinetics of DNA Repair. The kinetics of m^4T-1 and m^6G repair by mutant 56-8 were studied at 37 °C. Gels showing the time course of reactions with 0.10 pmol of protein and 0.13 pmol of m^4T-1 or m^6G substrate are presented in Figure 2, and the data obtained by phosphor image quantitation are plotted in Figure 3A. On the basis of the second-order rate equation (see Experimental Procedures), the $\ln[(B_0 - x)A_0/[(A_0 - x)B_0]$ versus time was plotted, as shown in Figure

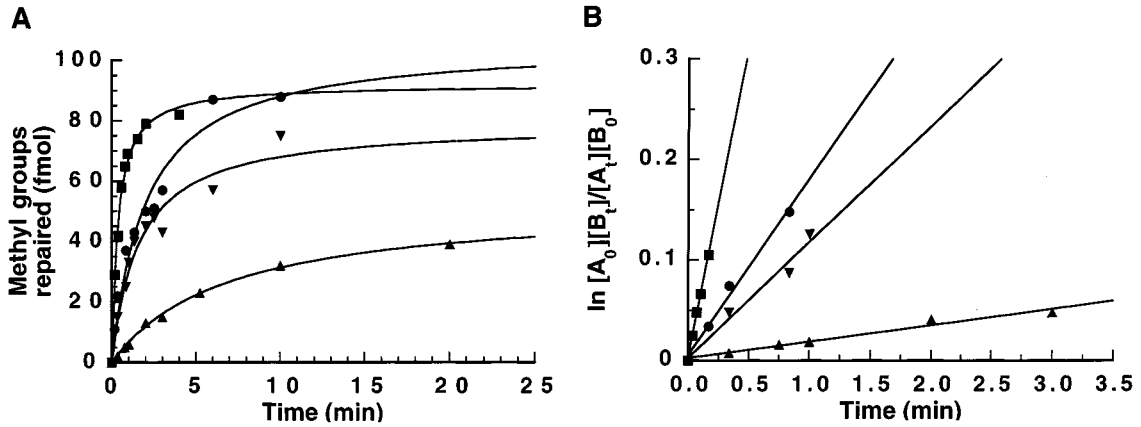

FIGURE 3: Kinetics of m[4]T-1 and m[6]G repair by purified WT and mutant 56-8 MGMT. (A) Time courses of repair obtained by phosphor image analysis of gels shown in Figure 2: (●) mutant 56-8 acting on m[4]T-1, (▼) mutant 56-8 acting on m[6]G, (▲) WT MGMT acting on m[4]T-1, and (■) WT MGMT acting on m[6]G. (B) The data depicted in panel A were replotted according to the second-order rate equation (see Experimental Procedures). A_0 was the initial concentration of substrate and B_0 the initial concentration of alkyltransferase, and A_t and B_t were the concentrations of substrate and alkyltransferase at time t, respectively. Symbols are as described for panel A.

Table 1: Mutant 56-8 and WT AGT Rate Constants (k)[a,b]

	k(m[6]G)	k(m[4]T-1)	k(m[4]T-1)/k(m[6]G)	k(m[4]T-2)	k(m[4]T-2)/k(m[6]G)
mutant 56-8	6.0×10^6	9.2×10^6	1.5	1.9×10^6	0.3
WT AGT	35.5×10^6 [c]	0.8×10^6	0.02	0.5×10^6	0.01
mutant/WT	0.2	11.5	75	3.8	30

[a] Rate constants were calculated from the slopes of the lines in Figure 3b, unless otherwise noted (units of M^{-1} s^{-1} at 37 °C). [b] The error in these values is estimated to be less than 20% on the basis of the variation of individual data points from the best-fit line in Figure 3B. [c] From Arrhenius plot (Figure 4).

3B. Because the slopes are equivalent to $k(B_0 - A_0)$, the rate constants could be calculated, and these values are shown in Table 1. Data for repair of the two substrates by WT MGMT under the same conditions are also shown in Figures 2 and 3; k(m[4]T-1) for the WT protein, calculated as described above, is shown in Table 1. The repair of m[6]G by WT MGMT was extremely rapid at 37 °C, reaching 50% completion within seconds. To verify the accuracy of this rate constant measurement, additional reactions were carried out at 31 °C, and at four lower temperatures. In these reactions, 0.10 pmol of WT MGMT was incubated with 0.20 pmol of m[6]G substrate for various times. The rate constants at 31, 25, 20, 14, and 10 °C were determined, and the log k for each was plotted as a function of the reciprocal of the absolute temperature in an Arrhenius plot (Figure 4). A linear relationship was observed, allowing us to estimate the k(m[6]G) at 37 °C for WT MGMT by extrapolation (Table 1). A similar approach for determining the k(m[6]G) for WT MGMT at lower temperatures has been described previously (*41*).

The foregoing measurements, summarized in Table 1, indicate that mutant 56-8 has a very different substrate preference compared to WT MGMT. The mutant repaired the m[4]T-1 substrate 11.5-fold more efficiently than the WT protein. The mutant repaired the m[6]G substrate nearly as well as m[4]T-1, although repair of m[6]G was >5-fold less efficient than that by WT MGMT. Importantly, the ratio k(m[4]T)/k(m[6]G) for mutant 56-8 was 75-fold greater than for WT MGMT.

Kinetics of Repair of a Second m[4]T Substrate. Since the efficiencies of many DNA repair processes are dependent upon sequences neighboring the damage (*42*), we carried out

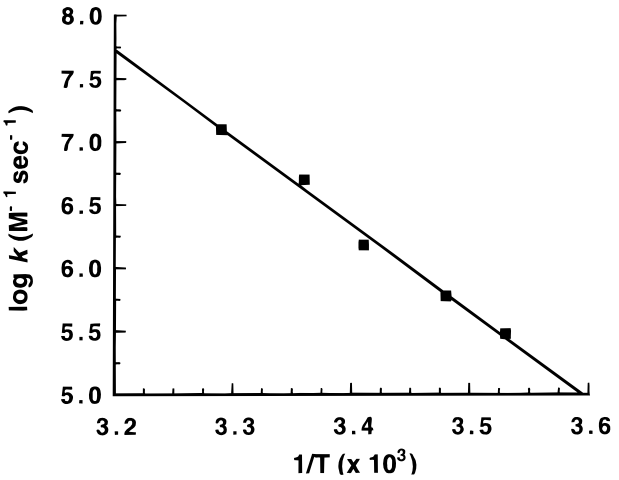

FIGURE 4: Relationship between the second-order rate constant (k) and temperature (T) for repair of m[6]G by WT MGMT. The plot was based on the Arrhenius equation $k = Ae^{-E_a/RT}$, which describes the temperature dependence of a specific rate constant. From this plot, the activation energy (E_a) for the reaction was calculated to be 132 kJ/mol.

additional kinetic studies using the same conditions, but with a different m[4]T-containing substrate, m[4]T-2. The time courses of repair by mutant 56-8 and WT MGMTs are shown in Figure 5, and the rate constants are listed in Table 1. Both MGMTs repaired the m[4]T-2 substrate less efficiently than m[4]T-1. The mutant was again more efficient than WT MGMT (3.8-fold), and the ratio k(m[4]T)/k(m[6]G) for the mutant was 30-fold greater than for the WT protein. The fact that the preference of mutant 56-8 for m[4]T was not as great with the m[4]T-2 substrate as with m[4]T-1 suggests a potential role for sequence context in the recognition of m[4]T by MGMT.

Competition Assay. To further compare the relative reactivities of WT MGMT and mutant 56-8 toward m[4]T residues in DNA, we used a competition assay where both m[4]T and m[6]G substrates were present simultaneously (*22*). The radiolabeled m[6]G substrate was added to all reaction mixtures at the same concentration. The unlabeled competitor m[4]T substrate (m[4]T-1) was added in increasing concentrations to inhibit repair of the m[6]G substrate. Reactions were initiated

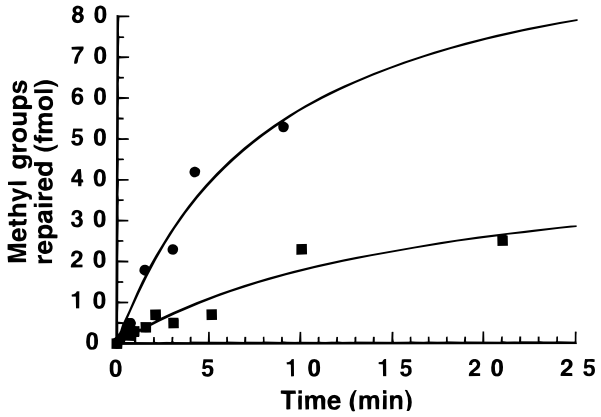

FIGURE 5: Kinetics of repair of m⁴T-2 by mutant 56-8 and WT MGMTs. Time courses of repair were obtained by using phosphor image analysis to quantitate electrophoretically resolved reaction products, as described in the legend of Figure 3: (●) mutant 56-8 and (■) WT MGMT.

by addition of substoichiometric amounts of MGMT and the mixtures incubated for 30 min at 37 °C. The molar excess of total substrate (m⁶G and m⁴T) was intended to render the assay more sensitive to competition; previous experiments had suggested that the specified conditions would permit reactions to proceed to near completion. As shown in Figure 6, increasing concentrations of the m⁴T substrate inhibited repair of the m⁶G substrate by both mutant 56-8 (panel A) and WT (panel B) MGMT, but importantly, the mutant was much more sensitive to inhibition. Data obtained by phosphor image analysis of the gel products are shown in Figure 7. For mutant 56-8, a substoichiometric concentration of m⁴T ($m^4T/m^6G = 0.6$) was required to inhibit repair of the m⁶G substrate by 50%. In contrast, a much higher concentration of m⁴T ($m^4T/m^6G = 166$) was necessary to yield 50% inhibition of the m⁶G substrate repair by WT MGMT.

DISCUSSION

Even when a crystal structure is available, our ability to predict the results of single-amino acid substitutions on the substrate specificity of enzymes is limited. Designing mutants with multiple substitutions is an even more daunting task, particularly if a conformational change in the protein occurs upon substrate binding. Directed molecular evolution techniques can circumvent these problems. By using positive genetic complementation, it is possible to select mutants with specific properties from large libraries of bacteria that express mutated enzymes. Screening can also identify mutants with desired characteristics, albeit less efficiently.

In the work presented here, we used directed molecular evolution to increase the activity of MGMT toward m⁴T, a relatively weak substrate for the WT protein. Several mutants previously selected in *E. coli* for their ability to protect against killing by MNNG and BG were screened for enhanced repair of synthetic oligonucleotides containing m⁴T at a single site. A mutant with increased activity toward m⁴T that contained eight amino acid substitutions (mutant 56-8) was identified by using bacterial lysates (Figure 1) and was purified for kinetic analysis.

The value of the second-order rate constant we obtained for repair of m⁶G by purified WT MGMT at 37 °C was 35.5 $\times 10^6$ M^{-1} s^{-1} (Table 1), in good agreement with previously

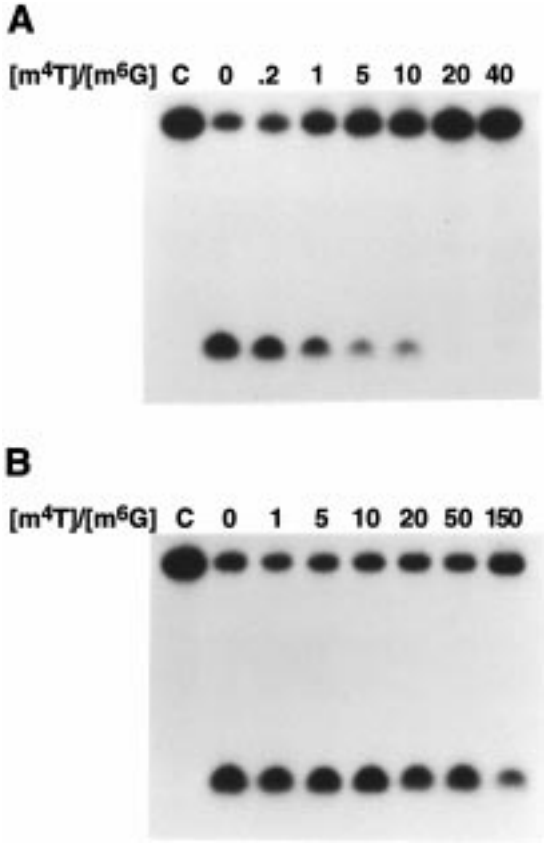

FIGURE 6: Competition assays. Inhibition of repair of m⁶G by m⁴T. WT MGMT or mutant 56-8 (0.23 pmol) was added to ^{32}P-labeled m⁶G substrate (0.30 pmol) in the presence of increasing concentrations of an unlabeled m⁴T-1 substrate. Reaction mixtures were incubated at 37 °C for 30 min. Following digestion with *Pst*I, samples were electrophoresed on a 20% polyacrylamide gel. Lane 1 in each autoradiogram shows a control reaction without protein: (A) mutant 56-8 and (B) WT MGMT.

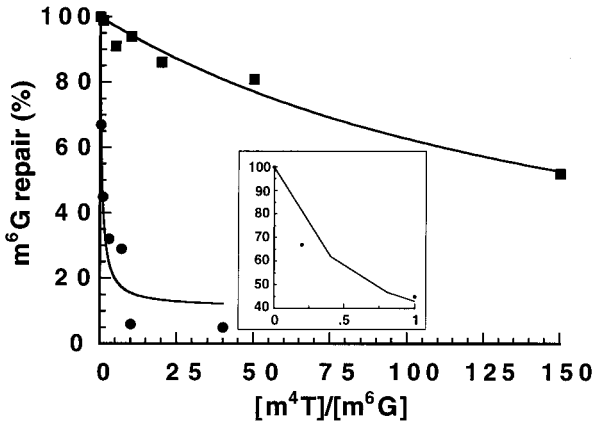

FIGURE 7: Quantitation of the competition between m⁴T-1 and m⁶G substrates for WT MGMT and mutant 56-8. The electrophoretically resolved reaction products shown in Figure 6 were quantitated by using phosphor image analysis. The level of repair of m⁶G was normalized to that observed in the absence of m⁴T-1 (Figure 6, lane 0): (●) mutant 56-8 and (■) WT MGMT. The inset shows inhibition at low m⁴T-1 concentrations observed for mutant 56-8.

reported values (*22*, *23*, *41*). The value of the rate constant for repair of a m⁴T substrate with a similar sequence (m⁴T-1) was 0.8×10^6 M^{-1} s^{-1}, demonstrating that WT MGMT repairs m⁴T 44-fold less efficiently than m⁶G in this assay. WT MGMT repaired a different m⁴T substrate (m⁴T-2) 71-fold less efficiently than m⁶G. m⁴T-1 is the more appropriate

substrate for comparison, however, because of its similarity to the m^{6}G substrate in sequence and in the position of the adduct with respect to the ends of the oligonucleotide substrate. Our values for relative substrate preference are in agreement with a previous report (*22*), in which WT MGMT repaired m^{6}G 35-fold more efficiently than m^{4}T. A greater relative preference for m^{6}G was observed in another study in which [^{3}H]-*N*-methyl-*N*-nitrosourea (MNU)-treated poly(dT) hybridized to poly(dA) was used as a m^{4}T substrate (*23*). In this work, WT MGMT repaired m^{4}T with 1/5000th the efficiency found for m^{6}G. Poly(dT)·poly(dA) does not assume the B-form structure dominant in DNA in solution, so the greatly reduced level of m^{4}T repair may have been due, at least in part, to poor recognition of the homopolymer duplex. The O^4-position of thymine is the most reactive toward MNU, but the presence of less frequently formed adducts at the O^2- and N3-positions may have also inhibited recognition and repair of m^{4}T. In summary then, our data are consistent with the general observations of others that m^{4}T is a substrate for WT MGMT, but is repaired less efficiently than m^{6}G.

The value of the second-order rate constant for repair of m^{4}T-1 by mutant 56-8 was 9.2×10^6 M^{-1} s^{-1} (Table 1), approximately 12-fold greater than that for the WT protein. The rate constant for repair of m^{6}G was only slightly lower at 6.0×10^6 M^{-1} s^{-1}. The enhanced m^{4}T repair was accompanied by a moderate decrease in the level of m^{6}G repair, the rate constant being approximately $^1/_6$ of that of WT MGMT. The ratio k(m^{4}T)/k(m^{6}G) for the mutant was increased 77-fold compared to the WT value, indicative of the very substantial change in substrate specificity.

Competition experiments with both m^{4}T and m^{6}G present in reaction mixtures suggested an even greater alteration (Figures 6 and 7). A [m^{4}T]/[m^{6}G] ratio of 166, i.e., a 166-fold molar excess of m^{4}T, was necessary to inhibit WT MGMT repair of m^{6}G by 50%, while a ratio of only 0.6 was required for 50% inhibition of m^{6}G repair by the mutant. The competition data thus indicate that mutant 56-8 has a 277-fold greater preference for m^{4}T over m^{6}G than the WT protein. Although the competition experiments provide convincing evidence of a change in substrate specificity, it should be pointed out that the relative amounts of m^{4}T used in these studies do not accurately represent an in vivo situation where DNA is exposed to a simple methylating agent. For example, under conditions of exposure to MNU or MNNG, approximately 100-fold less m^{4}T is formed than m^{6}G (*3*).

It is known that the surrounding sequence can modulate the efficiency of m^{6}G repair by WT MGMT, perhaps due to stacking interactions or steric hindrance. We observed less efficient repair of m^{4}T by both mutant and WT MGMT with the m^{4}T-2 substrate than with m^{4}T-1. Previous studies have shown that the base 5′ to the methylated nucleotide can affect the repair of m^{6}G by MGMT (*43*), although this bias was not observed for the bacterial alkyltransferase, Ogt (*44*). Substrates m^{4}T-1 and m^{4}T-2 both contain cytosine immediately 5′ of the methylated base, indicating that the decreased efficiency we observed for m^{4}T-2 may be due to other sequence differences. It has been shown that MGMT interacts with eight base pairs in DNA (*41*). Although sequence may play a role in the repair of the m^{4}T substrates by both WT MGMT and mutant 56-8, differences in the proximity of the modified base to the 5′-end of the oligonucleotides may also be involved in recognition and repair of the adduct.

All eight of the amino acid substitutions in mutant 56-8 may not be necessary for the alteration in specificity. The eight changes are at sites that are not evolutionarily conserved, which may explain why the protein can tolerate so many mutations. Interestingly, each of the eight changes is located at a position where sequence homology between WT MGMT and the *E. coli* alkyltransferase, Ada, is lacking (*18*). Seven of the changes are at positions where WT MGMT differs from the other *E. coli* alkyltransferase, Ogt. Although it is not consistent with previous reports (*45*), Ada was recently shown to have no preference for m^{6}G over m^{4}T (*22*). In contrast, Ogt was shown to repair m^{4}T 84 times faster than Ada (*46*), and reportedly has a preference for m^{4}T over m^{6}G (*21*, *45*, *46*). The bacterial alkyltransferases are similar to mutant 56-8 in that they are also resistant to BG (*47*, *48*). It is tempting to argue that we have created a mutant that is more like its bacterial counterparts, at least functionally, because it has better activity against m^{4}T and is resistant to BG. Unfortunately, we still lack a crystal structure for the human AGT with or without bound DNA.

It has been proposed that a m^{4}T·A base pair increases the degree of local curvature in DNA and that this may be responsible for the lower activity of WT MGMT on m^{4}T (*49*). It is possible that the active site of mutant 56-8 may accommodate an increased degree of curvature, and that the structural changes permitting this accommodation can also explain the decreased efficiency in repair of m^{6}G.

On the basis of the strong mutagenic potential of m^{4}T, and the fact that it is a more persistent adduct than m^{6}G, it is possible that m^{4}T is as biologically significant as or even more biologically significant than m^{6}G. The mutagenicity of different DNA alterations produced by alkylating agents could be important in the emergence of secondary leukemias after treatment of patients with chemotherapeutic alkylating agents (*50*). Use of human MGMT has been proposed for the protection of bone marrow against these drugs (*51*). Mutant MGMTs that enhance repair of specific lesions (e.g., m^{4}T) offer an advantage if the specific lesions are responsible for the toxic, mutagenic, or carcinogenic effects of a particular alkylating agent. If the initial mutations responsible for the secondary leukemias could be uncovered, mutant MGMTs that repair the critical lesion(s) more efficiently could be created, and gene therapy could be used to introduce such mutant enzymes to patients at risk.

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