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Specificity of DNA Repair Methyltransferases Determined by Competitive Inactivation with Oligonucleotide Substrates: Evidence That *Escherichia coli* Ada Repairs O^6-Methylguanine and O^4-Methylthymine with Similar Efficiency†

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ABSTRACT: DNA repair methyltransferases (MTases) are stoichiometric acceptor molecules that are irreversibly inactivated in the course of removing a methyl group from O^6-methylguanine (meG)-DNA or O^4-methylthymine (meT)-DNA. A new assay has been developed to determine the relative efficiency of repair of meG and meT. The assay is based on the deprotection of methylated restriction sites in synthetic oligonucleotides and can be used to measure meG repair or meT repair directly. More importantly, relative repair efficiencies can be measured in competition experiments, using each of the methylated oligomers in turn as an inhibitor of repair for the other. Relative repair rates are determined by numerical solution of the coupled rate equations that describe this competition to the experimental data. We find that the human MTase repairs meT about 35-fold less well than meG, qualitatively similar to earlier studies. Contrary to previous reports, however, we find that *Escherichia coli* Ada repairs meG and meT with nearly equal efficiency. This finding, in conjunction with other recent reports, may indicate that low meT repair is a relatively unusual characteristic of the human homolog.

DNA methylguanine methyltransferases (MTases)[1] demethylate (or, more generally, dealkylate) certain premutagenic adducts caused by exposure to alkylating agents. Curiously, repair methyltransferases are not true enzymes since they become inactivated in the course of repairing a single methylated base. This inactivation occurs because the methyl group that is removed from DNA is irreversibly transferred to a cysteine side chain in the active site of the protein (Lindahl et al., 1982; Olsson & Lindahl, 1980). Thus, DNA repair by this protein is formally a bimolecular chemical reaction (protein + me-DNA → me-protein + DNA). Published kinetic analyses are consistent with a second-order reaction (Chan et al., 1993; Graves et al., 1989; Zak et al., 1994).

O^6-Methylguanine (meG) is the most abundant adduct repaired by MTases, but a rarer methylation product, O^4-methylthymine (meT), is also a substrate (Graves et al., 1989; Sassanfar et al., 1991). Both meG and meT lead to mutations. meG can mispair with thymine, resulting in a GC to AT transition after the second round of replication; meT mispairs with guanine, resulting in a TA to CG transition (Dosanjh et al., 1991; Loechler et al., 1984; Swann, 1990).

Given the mutagenicity of meT, it is notable that many MTases have been reported to have a poor capacity to repair this adduct. For example, reports on the ratio of meG to meT repair activity for *Escherichia coli* Ada (or for its methylguanine methyltransferase domain) have ranged from about 20 to as high as 10 000 (Sassanfar et al., 1991; Zak et al., 1994). The wide range in these estimates is probably due to differences in the assays and substrates used.

One way in which variable results might arise is if the meG and meT substrates differ in some way other than simply the identity of the methylated base. For example, Zak et al. (1994) used DNA methylated by the addition of [^{3}H]-methyl-*N*-nitrosourea (MNU) as a substrate. Since guanine is methylated more readily than thymine, most of the O-methylated bases available to the methyltransferase are meG. To obtain a suitable meT substrate, poly(dT) was methylated with [^{3}H]MNU and then annealed to poly(dA). Using these two different substrates, a meG/meT preference of over 5000 was obtained for human MTase (Zak et al., 1994). However, the structure of poly(dA·dT) DNA is known to differ somewhat from classical B-form DNA (Arnott et al., 1983; Nelson et al., 1987), whereas the more heterogeneous DNA sequences used to assay meG transfer activity can be expected to be more B-like on average. It is possible that the very low meT repair activities measured by this and similar assays are due to poor recognition of poly(dA·dT) DNA rather than low reactivity with meT *per se*.

The potential for substrate artifacts exists even with oligonucleotides of defined sequence. Several groups have used self-complementary 12-mers containing a single meG or meT (Dolan et al., 1988b; Graves et al., 1989; Wilkinson et al., 1989). Wilkinson et al. (1989) found that *E. coli* Ogt prefers meG by a factor of 138, and both they and Graves et al. (1989) found that *E. coli* Ada repairs meG 10 000-fold more readily than meT. These results are in contrast

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[1] Abbreviations: MTases, methyltransferases; meG, O^6-methylguanine; meT, O^4-methylguanine; MNU, methyl-*N*-nitrosourea; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PCR, polymerase chain reaction; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; BSA, bovine serum albumin.

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to the observations of Sassanfar *et al.* (1991), obtained by a very different assay, which suggested that Ogt actually has a slight preference for meT and that Ada has only a 22-fold preference for meG. Possibly, use of the self-complementary dodecamers overestimates the meG/meT repair ratio somehow. The meG and meT bases in these substrates are each only four bases from the ends of the oligomers and it is possible that the two methylated bases affect the stability of the ends of the oligomer in different ways. Furthermore, both substrates have the potential to form a stem and loop structure instead of the desired duplex, and it may be that the meT substrate forms this structure to a greater extent. In this regard, it is worth noting the observation of one group that repair of the self-complementary meT oligomer could be detected only if the methylated oligomer were hybridized with excess unmethylated DNA of the same sequence (Dolan et al., 1988b).

Here, we report on the development and use of a competitive inactivation assay that avoids some artifacts possibly associated with certain earlier assays. Surprisingly, we find that the alkylguanine repair domain of Ada is able to repair meG and meT with nearly equal facility. Human MTase repairs meG about 35 times more efficiently than meT, consistent with the conclusions of others.

EXPERIMENTAL PROCEDURES

Sequences. PCR primers for cloning *ada* were developed from the published sequence (Nakabeppu et al., 1985): Ada 5′,5′ CCCTATGGATCCCAATTCCGTCACGGTGGC 3′ (designed to prime from glutamine 179); Ada 3′,5′ CCCTATGAATTCTTACCTCTCCTCATTTTC 3′. The substrate duplexes contain three-base 5′ TTT overhangs on each strand to allow radiolabeling with DNA polymerase and [α-^{32}P]-dATP; in practice, however, we have always labeled the substrates by phosphorylating the 5′ end of the methylated strand. The meG substrate is

```
5' TTTAGCTGCACAGACTGCCGG*CGCTGCAGGTCGACTAGC 3'
   3' TCGACGTGTCTGACGGCCGCGACGTCCAGCTGATCGTTT 5'
```

G* is meG for the methylated substrate or guanine for the control substrate; the underlined sequence is the *Hha*I recognition site. The meT substrate is

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5' TTTAGCTGCACAGACTGCCGGAT*CCGCTGCAGGTCGACTAGC 3'
   3' TCGACGTGTCTGACGGCCTAGGCGACGTCCAGCTGATCGTTT 5'
```

T* is meT in the methylated substrate or thymine in the control substrate; the underlined sequence is the *Bam*HI recognition site.

Strains. BL21(DE3) *ada*$^-$ *ogt*$^-$, a derivative of BL21-(DE3) (Studier et al., 1990), was constructed in two steps by P1 transduction (Miller, 1972) using phage lysates grown on GW5352 (*ada-10*::Tn10) (Lemotte & Walker, 1985) and GWR107 *ogt-1*::kanR (Rebeck & Samson, 1991). BL21-(DE3) *ada*$^-$ *ogt*$^-$ is resistant to tetracycline and kanamycin and highly sensitive to the killing effects of MNNG (data not shown). GW5352 and GWR107 were gifts of Dr. Leona Samson.

Proteins

Cloning of the ada Methyltransferase Domain and Expression and Purification of the Protein. The *ada* gene was cloned by the polymerase chain reaction (PCR) from genomic DNA. Genomic DNA was prepared from GWR107 using standard procedures (Ausubel et al., 1992). The PCR fragment was subcloned into pRSETA (Invitrogen), which contains a 5′-polyhistidine coding region. The gene, including the polyhistidine tag, was then subcloned into pG5, a T7 expression vector (Alexander et al., 1992). The resulting plasmid is called pH_6Ada. The gene was then sequenced using the dideoxy chain-termination method (Sanger et al., 1977).

BL21(DE3) *ada*$^-$ *ogt*$^-$ containing pH_6Ada was grown in 0.5−2.0 L of LB in the presence of 100 μg/mL ampicillin to an A_{600} of 0.8, at which time IPTG was added to a concentration of 0.4 mM. Cultures were grown for another 2−3 hours, and the cells were spun down and frozen for later use. All subsequent purification steps were done at 4 °C using buffers purged with helium prior to use. Cell pellets were resuspended in column binding buffer (20 mM Tris, pH 8, 0.5 M NaCl, and 5 mM imidazole), sonicated (Branson Sonifier 450, microtip, 50% duty cycle, 7 output, for 3−4 times 5 cycles), and centrifuged at 11000*g* for 10 min. The supernatant was loaded onto a Ni-NTA-agarose column (Qiagen) that had been preequilibrated with 3 column volumes of deionized water, 5 volumes of charge buffer (50 mM $NiSO_4$), and 3 volumes of binding buffer. A column volume of 0.5 mL was sufficient to purify 10 mg of protein. After the supernatant was loaded, the column was washed with several volumes of loading buffer, and then eluted with 2 volumes each of 20 mM, 40 mM, 60 mM, 100 mM, 500 mM, and 1 M imidazole in 20 mM Tris, pH 8, and 0.5 M NaCl. DTT (1 mM) and 5% glycerol were added to column fractions and the purity of each fraction was assessed by SDS−polyacrylamide gel electrophoresis. Typically, the most pure fraction was the one that eluted at 100 mM imidazole. Purified fractions were aliquoted and frozen at −80 °C.

Human Methyltransferase. Human methylguanine methyltransferase was a gift from Dr. Phil Potter.

Oligonucleotide Substrates

Synthesis and Purification of Oligomers. Oligomers were synthesized on an Applied Biosystems, Inc., 392 DNA synthesizer. In general, oligomers with no modified bases were cleaved from the support resin with ammonium hydroxide, deprotected overnight at 55 °C, and purified and detritylated on OPC cartridges (Applied Biosystems) according to manufacturer's directions. They were then lyophilized and redissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). DNA synthesis chemicals, including meG and meT phosphoramidites, were purchased from Glen Research. The meG oligomer was synthesized as usual except it was cleaved from the solid support and deprotected using a 10% solution (v/v) of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) in methanol. The deprotection was allowed to proceed in the dark at room temperature for several days, at which time the oligomer was purified and detritylated as described above. The meT substrate was synthesized as for a normal oligonucleotide, except the capping reaction was eliminated for meT and all subsequent additions (Sassanfar et al., 1991). Cleavage and deprotection was achieved as described for the meG oligomer. After 3 days of deprotection, the DNA was ethanol-precipitated from

the DBU mixture, and purified and detritylated on an OPC cartridge. Because the capping reaction was not used throughout the synthesis, the meT oligomer was further purifed on a 10% acrylamide (29:1 acrylamide:bisacrylamide) and 6 M urea gel. The desired product was identified by UV shadowing and was then eluted in 0.5 M ammonium acetate and 1 mM EDTA overnight at 37 °C. The oligomer was desalted with a Sep-Pak reverse phase cartridge (Millipore) and eluted in 50% acetonitrile. The eluate was lyophilized, dissolved in water, and reprecipitated with ethanol.

Radiolabeling of Oligomers. Oligomer (1 pmol) was incubated with 10 μCi of [γ-^{32}P]ATP (Amersham) and 10 units of T4 polynucleotide kinase (New England Biolabs) in a volume of 15 μL for 30 min at 37 °C. The kinase was inactivated by adding EDTA to 13 mM and incubating the mixture at 65 °C for 20 min, and nonradioactive ATP was added to a final concentration of 58 μM. A slight excess of the complementary strand (1.2 pmol) was added and the oligomers were annealed by heating to 95 °C and slowly cooling. The oligomers were separated from ATP either by ethanol precipitation or by using a G-25 spin column (Boehringer Mannheim) followed by ethanol precipitation. The precipitated radiolabeled DNA (nominally 10^{-12} mol) was redissolved in TE containing 10^{-11} moles of annealed unlabeled duplex, and dilutions of this stock were made as appropriate.

Assay

Competition Assay. Increasing dilutions of each protein preparation were incubated with radiolabeled meG or meT substrate (2×10^{-9} M) in reaction buffer (Tris-HCl (pH 8.0), 0.2 mg/mL BSA, and 1 mM DTT) for 30 min at room temperature in a volume of 10 μL. The samples were then digested with 20 units of *Hha*I (meG substrate) or *Bam*HI (meT substrate) in the presence of 100 ng/μL herring sperm DNA (Promega) and the appropriate supplied buffer (New England Biolabs). After electrophoresis of the samples on a 12% nondenaturing acrylamide gel, gels were dried and placed on a PhosphorImager screen (Molecular Dynamics) for quantitation. The dilution of protein that yielded between 60% and 90% of the maximum reaction observed at the highest protein concentrations was used in the competition experiments. Previous kinetic experiments had indicated that an incubation time of 30 min was sufficient for these purposes (data not shown). The competition experiments were performed essentially as described above, except that varying amounts of competing unlabeled oligomer were also added.

Quantitation. A Molecular Dynamics PhosphorImager was used to quantitate the extent of repair of the radiolabeled substrate. The intensity of each band was obtained using Molecular Dynamics ImageQuant 3.3 software and was background-corrected either by integrating an identically shaped object in each lane or by using the background feature of the software to obtain an average value between the cut and uncut bands in all lanes. In some assays, experimental values were further normalized to controls to take into account (i) repaired substrate that might escape restriction digestion and (ii) unrepaired substrate that might nevertheless contribute to the digested substrate band. The relevant reactions were not run for all experiments since the effect of these corrections was minor.

Data Fitting. Rate constants for meG repair and for meT repair were calculated by numerically integrating the equations that describe the rate of production of repaired DNA as a function of time (t) and fitting these solutions to the observed extent of repair after 30 min in the presence of varying amounts of competitor DNA. The equations describing the rate of production of unmethylated G and of unmethylated T are coupled because repair of either meG or meT decreases the concentration of active protein available for repair of both. Thus

$$\frac{d[G]}{dt} = k_{meG}([P]_0 - [G] - [T])([meG]_0 - [G])$$

$$\frac{d[T]}{dt} = k_{meT}([P]_0 - [G] - [T])([meT]_0 - [T])$$

where $[meG]_0$ and $[meT]_0$ are the initial concentration of the meG and meT substrates, respectively, k_{meG} and k_{meT} are the rate constants, [G] and [T] are the concentrations of repaired substrates, and $[P]_0$ is the initial concentration of active protein. Numerical integrations and parameter estimates were performed using MLAB (Civilized Software).

RESULTS

Cloning and Expression of Ada. The MTase domain of *E. coli* Ada (residues 179−354; hereafter called Ada-C) was cloned by PCR from an *E. coli* K12 strain as described in Experimental Procedures and was shown to be identical to the reported sequence (Nakabeppu et al., 1985). Ada-C was expressed and purified as a His-tagged fusion in a strain lacking endogenous MTase activity. Protein preparations were >90% pure.

Principle of the Assay. Some restriction enzymes will not cut their cognate sites if the site contains meG or meT (Richardson & Richardson, 1991; Voight & Topal, 1990). The extent of repair in substrates that contain methylated restriction sites can therefore be determined by their sensitivity to restriction digestion. We synthesized two oligonucleotide substrates with methylated bases. The meG substrate is a duplex 39mer with the methylated base located within a *Hha*I restriction endonuclease recognition site. The meT substrate is a duplex 42mer with a meT base within a *Bam*HI restriction endonuclease site. The sequences of the two substrates differ only in the insertion of three bases required to create the *Bam*HI site in the meT substrate. Both methylated bases are at least 19 nucleotides away from each end of the substrate.

In order to compare the relative reactivities of the substrates directly, we developed an assay in which meT and meG repair occur in the same tube. One of the substrate oligomers (meG in Figure 1) is radiolabeled and is used at a constant concentration in each reaction, while unlabeled competitor (meT) is added in varying amounts to the substrate. Methyltransferase is then added to this mixture and allowed to react. As shown in Figure 1, increasing concentrations of competitor result in a decrease in the amount of repaired substrate, as determined by restriction digestion of the radiolabeled oligonucleotide.

It is important that the concentration of methyltransferase not be vastly greater than the concentration of substrate because then the presence of competitor will have only a very slight effect on the extent of substrate repair. In fact,

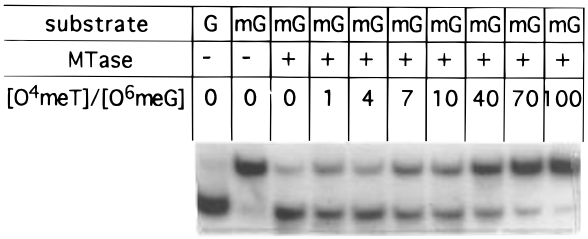

FIGURE 1: Typical autoradiogram from a competition experiment using human MTase. A substoichiometric amount of MTase was added to radiolabeled methylguanine-DNA (mG) in the presence of varying amounts of O^4MeT competitor as described in the Experimental Procedures section. After repair, the DNA was digested with *Hha*I endonuclease, electrophoresed on a 12% polyacrylamide gel, and exposed to film. Control lanes show (first lane) the gel mobility of unmethylated DNA (G) digested by *Hha*I and (second lane) the mobility of methylated substrate treated with *Hha*I but not digested because it has not been repaired by MTase. High O^4MeT to O^6MeG ratios are required to inhibit the repair reaction by the human protein.

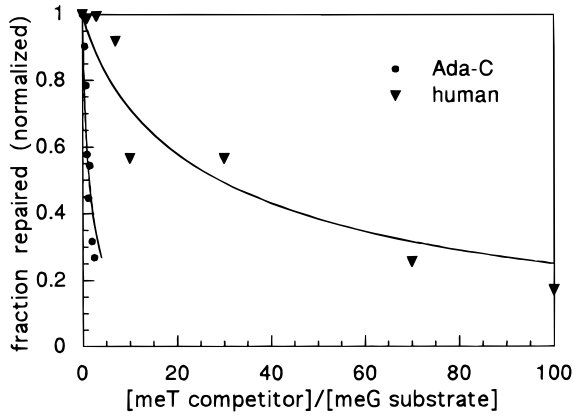

FIGURE 2: Effect of meT competitor on the repair of meG by Ada-C and human MTase. Significantly lower concentrations of meT oligonucleotide are required to inhibit meG repair by Ada-C (●) than by human MTase (▼). The fraction repaired for each data point has been normalized to the amount of repair in the absence of inhibitor. This normalization allows a direct comparison of the data from the two proteins. The curves were calculated numerically using values for k_{meG} and k_{meT} that were obtained by fitting the rate equations to the unnormalized data and then normalizing to the calculated fraction that is repaired in the absence of competitor. Values for P_0 used in the calculation were obtained by titration of the proteins against a known concentration of substrate.

the assay is expected to be most sensitive when the protein concentration is close to that of the substrate. When only one radiolabeled substrate is used, the analysis of the data is most straightforward when the protein concentration is actually slightly below that of the substrate. An amount of protein was therefore used that repairs most, but not all, of the substrate in the course of the reaction even in the absence of competitive inhibitor. Alternatively, as shown below, it is less critical to perform the assays at concentrations very close to stoichiometric equivalence if two sets of assays are performed in parallel, one using radiolabeled meG as the substrate and unlabeled meT as the competitor and the other using radiolabeled meT as the substrate and unlabeled meG as the competitor. Moreover, if the assays are performed at more than one concentration of protein, then the concentration of active protein can be determined from the fit.

Competitive Inactivation of meG Repair by meT. Ada-C and human MTase were assayed using radiolabeled meG oligomer mixed with increasing amounts of unlabeled meT substrate. A typical autoradiogram of such an assay is shown in Figure 1. Figure 2 shows the results of such assays for both methyltransferases. For Ada-C the amount of meT oligomer required to reduce meG repair by 50% is very nearly equal to the amount of radiolabeled meG substrate. We infer from this that the efficiency with which Ada repairs (and is inactivated by) meT is nearly the same as the efficiency with which it repairs (and is inactivated by) meG. In contrast, the human protein requires a greater than 30-fold molar excess of meT in order to achieve 50% inhibition. Therefore, unlike Ada-C, meG repair by the human protein is only weakly affected by meT competition.

For a quantitative analysis of the data, we solved the rate equation for k_{meG} using the uninhibited reaction and then fit k_{meT} to the data obtained in the presence of competitor. The values for $[P]_0$ (initial concentration of active protein) used in these calculations were based on titration of each protein against a known concentration of methylated substrate; at dilutions that resulted in incomplete repair of DNA after prolonged incubation, it was assumed that the protein concentration was limiting. The protein cannot be completely reacted because that would imply an infinitely high rate constant. For the sake of calculating rate constants, it was assumed that 99% of the protein had reacted under these conditions. (While the exact values for k_{meG} and k_{meT} depend on the amount of protein reacted, the ratio of the two rate constants is rather insensitive to this assumption.) The quantitative fits confirm the qualitative conclusions reached above. The rate constant for repair of meG by Ada is only 1.5 times greater than is the rate constant for repair of meT. In contrast, the human protein has a k_{meG}/k_{meT} ratio of 36.5. The second-order rate constants for repair of meG by this assay are 5.0×10^6 M^{-1} s^{-1} for Ada-C and 5.3×10^6 M^{-1} s^{-1} for the human protein, although, again, these values depend on the assumption of 99% reacted protein and are given for comparative purposes only.

Simulated Reciprocal Inhibition Assays and the Use of Protein Concentration as a Variable. If both meT inhibition of meG repair and meG inhibition of meT repair are measured, it is unnecessary for methyltransferase to be substoichiometric. To see why this is so, we calculated theoretical inhibition curves by solving the rate equations (see Experimental Procedures) under conditions of equivalent and excess protein (Figure 3). The curves in Figure 3A were calculated for a hypothetical MTase that has identical rate constants for the two methylated bases ($k_{meG} = k_{meT}$). Note that even when the protein concentration is equal to the substrate concentration ($[P]_0 = [S]_0$), an excess of competitor over substrate is required to achieve a 2-fold reduction in the fraction of substrate repaired compared to the uninhibited reaction. Furthermore, the amount of competitor that is required to achieve this degree of inhibition increases with protein concentration. An equimolar amount of competitor yields a 50% reduction in product only when $[P]_0 \ll [S]_0$ and $k_{substrate}$ is sufficiently high that nearly all the protein reacts.

Since the rate constants for meG and meT repair are assumed to be identical in Figure 3A, the same curve is found whether the substrate is meG and the competitor is meT or the other way round. What makes the competition assay useful is that the curves are sensitive to differences in k_{meG} and k_{meT} even at protein concentrations that are not in close stoichiometric equivalence to substrate concentrations. The

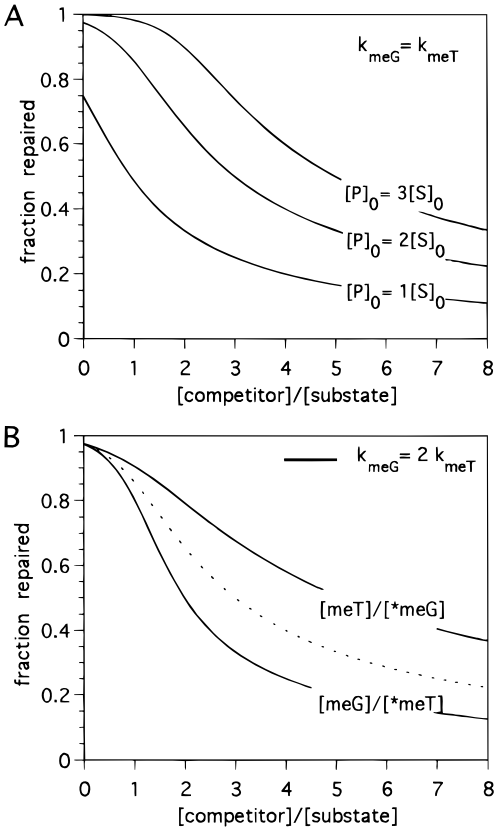

FIGURE 3: (A) Theoretical curves showing the effect of increasing protein concentration on the amount of competitor required to inhibit the reaction. The curves were calculated for a hypothetical MTase for which $k_{meG} = k_{meT}$. The value for the rate constants was chosen such that 75% of the substrate is repaired after 30 min when the initial protein concentration is equal to the initial substrate concentration ($[P]_0 = [S]_0$) and there is no inhibitor present. (B) Theoretical curves calculated for a 2-fold excess of protein over substrate. The dashed line is the same as the middle line in panel A and is for a hypothetical MTase for which $k_{meG} = k_{meT}$. The solid lines are for a hypothetical MTase for which $k_{meG} = 2k_{meT}$. [meT]/[*meG] indicates that meG is the substrate and meT is the competitor. [meG]/[*meT] indicates that meT is the substrate and meG is the competitor.

dashed line in Figure 3B shows the curve expected when $[P]_0 = 2[S]_0$ and $k_{meG} = k_{meT}$ and is the same curve as the middle curve in Figure 3A. The solid lines in Figure 3B show the effect of a 2-fold lower value for k_{meT} on assays performed using meG (top line) or meT (bottom line) as the substrate. Clearly, a difference in rate constants as small as 2-fold can be readily discerned in this type of assay even when protein is in moderate excess.

Reciprocal Inhibition Assays of Ada-C. To confirm the finding that Ada-C is only slightly more reactive toward meG than meT, we repeated the competition assays using both meG and meT as the substrates (Figure 4). In addition, these assays were done at two different dilutions of protein, one 33% more concentrated than the other, in order to better determine the initial concentration of active protein. It is immediately clear that the results are qualitatively consistent with a small difference in rate constants because the amount of meT required to inhibit meG repair is only slightly greater than the amount of meG required to inhibit meT repair. This is true at both protein concentrations. As expected from the simulated inhibition curves, greater amounts of each inhibitor are required when the protein concentration is higher.

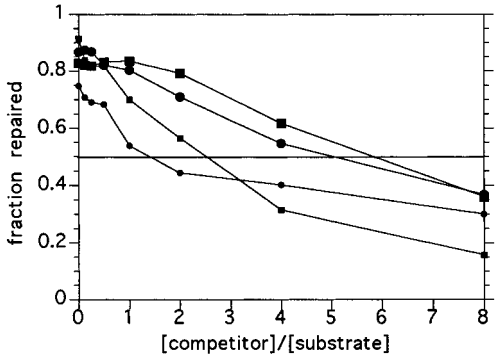

FIGURE 4: Reciprocal inhibition assays of Ada-C. Circles (both large and small) indicate radiolabeled meT substrate and unlabeled meG competitor. Squares (both large and small) indicate radiolabeled meG substrate and unlabeled meT competitor. Small symbols indicate the lower protein concentration (fit value = 4 nM). Large symbols indicate a dilution of protein that is 1.33 times more concentrated. The intersections of the data curves with the horizontal line drawn at the 50% repair level are the interpolated values used in data fitting.

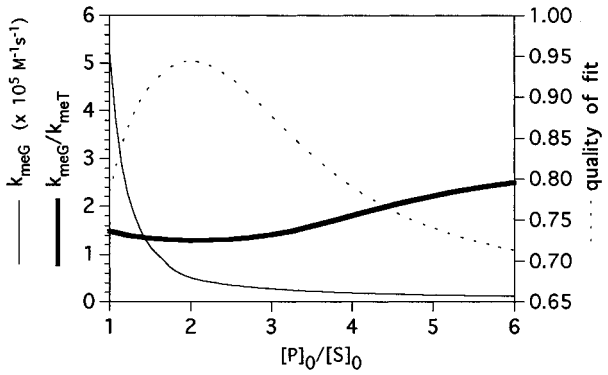

FIGURE 5: Sensitivity of k_{meG} and the ratio k_{meG}/k_{meT} to protein concentration for the reciprocal inhibition assays of Ada-C. Data fits were done at a series of assumed protein to substrate ratios ($[P]_0/[S]_0$) as described in the text. The heavy line is the ratio of rate constants, and the light line shows the value for k_{meG}. The quality of the fit is shown by the dashed line and is defined as $1 - \sum(M_{fit} - M_{obs})^2$. The summation is over the four interpolated data points from the experiments shown in Figure 4. M_{fit} is the expected value for the concentration of competitor that results in 50% repaired substrate based on the fitted kinetic parameters; M_{obs} is the observed value of competitor that results in 50% repaired substrate as obtained by interpolation of the nearest data points.

As with the first set of experiments, we confirmed the qualitative interpretation by fitting the data to the rate equations given in the Experimental Procedures section. For each of the four sets of assays in this experiment, the competitor concentration at which 50% of the substrate was repaired was obtained by interpolation between the experimental data points flanking the 50% repair level. These four values were then used to fit k_{meG} and k_{meT} simultaneously at different assumed ratios of protein to substrate ($[P]_0/[S]_0$). An additional constraint was imposed in the fitting in order to have the value of k_{meG} result in 90−95% repair in the absence of inhibitor, consistent with the experimental results. (Without this additional experimental constraint, the fit is underdetermined and the fits improve indefinitely with higher assumed values of $[P]_0$.)

Figure 5 shows the value of k_{meG} and the ratio of k_{meG} to k_{meT} as a function of the stoichiometry of protein to substrate for the more dilute protein solution. The best fit to the data (peak in the dashed line) gives a k_{meG}/k_{meT} ratio of 1.3 and occurs when the protein concentration is in 2-fold molar

excess over substrate ([S] = 2 nM, $[P]_0$ = 4 nM). Note that the ratio of rate constants for the two substrates is rather insensitive to the protein concentration, varying only between 1.3 and 2.2 over a 6-fold range in protein concentration. Thus, small errors in the data or the fitting are unlikely to produce very different conclusions regarding the relative reactivity toward meG and meT. As an example, the concentration of active protein obtained by this fit is severalfold lower than the expected value based on the optical absorbance of the protein preparation (data not shown). Whether this discrepancy reflects imprecision in the fit, the existence of inactive protein, or the presence of contaminating UV-absorbing materials, the sensitivity analysis shown in Figure 5 suggests that the calculated ratio of rate constants is not significantly affected by this uncertainty. The absolute rate constants, however, are quite sensitive to protein concentration, dropping more than 10-fold as the protein to substrate ratio increases from 1 to 2.

It is not entirely clear why the meG and meT curves intersect in Figure 4. Taking this result at face value, it appears that meG is a less effective competitor than meT at high concentrations despite the fact that k_{meG} is slightly greater than k_{meT}. One trivial explanation for the intersection of these curves is underestimation of the background correction in the assays using meT as the substrate. Uncorrected background counts that are present in both the repaired and unrepaired bands will have the effect of making the apparent "fraction repaired" for each data point closer to 0.5 than it should be. The competitor concentrations at which close to half the substrate is repaired are the most accurate because they are the least affected by errors of this type, which is one reason for using the 50% repair value in the quantitative fits.

DISCUSSION

Our conclusion that *E. coli* Ada (or Ada-C) reacts almost as well with meT as it does with meG is strikingly different from the conclusion reached by others. Previous studies have suggested that Ada reacts between 22 and 10 000 fold more efficiently with meG. The wide range in reported substrate preferences must reflect differences in the assays used, and some of these were described in the introduction. In some ways, the most important findings to reconcile with our own observations are those of Sassanfar et al. (1991), who reached similar conclusions for the human protein, but who found that Ada has a 22-fold preference for meG. Their assay was also based on inactivation of methyltransferase using synthetic oligonucleotides. Oligonucleotides containing either a single meG or a single meT were allowed to react with methyltransferase for 15 min, at which time an excess of ^{3}H-methylated high molecular weight DNA was added. The concentration of methylated oligonucleotide required to reduce the transfer of ^{3}H-methyl groups to the protein by 50% was taken to be a measure of the reactivity of the protein with the methylated oligonucleotide. One possible explanation for the difference in Ada activity is that the meT base is only five bases from the end of the oligonucleotide in their substrate, while meG is seven. Perhaps Ada is unusually sensitive to the proximity of its substrate nucleotide to the end of the oligomer.

Whether the assay described here provides a truer measure of substrate specificity is uncertain, of course, as there may be artifacts in this assay that have escaped our attention. In principle, however, the assay has several advantages. First, it relies on competitive inactivation of the protein with both substrates in the same tube at the same time. Second, repair of meT is measured directly, rather than relying on inactivation of meG repair activity. Third, the substrates are similar in sequence and in length, and the methylated bases are at least 19 bases from each end.

Previous studies of alkylguanine repair have found the effect of sequence context to be relatively small. For example, when the nucleotide 5′ to the methylated base was C rather than G, extracts from human cells repaired roughly twice as much meG in the same amount of time.(Dolan et al., 1988a) On the other hand, no such preference for meG repair was observed for the *E. coli* methyltransferase Ogt (Vidal et al., 1997). It is possible, of course, that repair of meT is more sensitive to sequence context than is repair of meG, and perhaps it even has a different specificity. If this turns out to be true, assays that are based on defined oligonucleotides will be meaningful only if a large number of different sequences are examined.

One result that remains unexplained is the crossing of the meG inhibition and meT inhibition curves in Figure 4. As discussed in the Results section, this could be due to errors in background correction. However, it is also possible that the methyltransferase reaction is more complicated and that assumptions about second-order kinetics and competitive inactivation are not completely valid. One way in which the reaction could deviate from pure second-order kinetics is if methylated protein (a product of the reaction) binds to substrate in competition with active protein. The shapes of the inhibition curves could differ if the methylated protein binds with different affinities to the two substrates.

It has been generally thought that *E. coli* Ogt is unusual among DNA repair methyltransferases in its similar repair rates for meT and meG. However, we have shown here that Ada-C also has similar reactivity toward meT and meG, and it has recently been shown that the same is true for mouse MTase (Kawate et al., 1995). The ability of mouse MTase to efficiently repair meT was confirmed *in vivo* using an *E. coli lacZ* reversion assay (Kawate et al., 1995). Some other mammalian homologs may also be more capable of meT repair activity than is the human protein. Rat MTase has about a 27-fold lower meG/meT repair preference than does the human protein, as assayed using ^{3}H-methylated DNA substrates (Zak et al., 1994). If one accepts our value of 36.5 for the meG/meT preference of the human protein, this implies a meG/meT preference of only 1.3 for the rat protein. [The experimental preference reported for the rat protein is much greater (Zak et al., 1994), perhaps because of differences in the two substrates, as we outlined in the introduction.] Clearly more studies are required, but meT repair proficiency *in vitro* may yet prove to be more the rule than the exception.

The relevance of our assays to DNA repair activity *in vivo* remains unclear. If *E. coli* Ada and Ogt each repair meT and meG with roughly equal proficiency [as suggested here for Ada and by Sassanfar et al. (1991) for Ogt], then we would expect the combined *in vivo* repair of these adducts to be similar as well. Perhaps arguing against this prediction is the observation of Dosanjh et al. (1991) that meT is much more mutagenic than meG when incorporated into a plasmid in a site-directed manner. On the other hand, this assay is

affected by the efficiency and fidelity of replication opposite the methylated bases, as well as by various DNA repair pathways. Although *in vitro* experiments with the Klenow fragment of Pol I suggest that meT and meG miscode with similar efficiency (Dosanjh et al., 1990; Singer et al., 1989), replication *in vivo* may be more complicated. For example, the mutagenicity of meG is apparently reduced by replication blocks mediated by the mismatch repair system (Pauly et al., 1994, 1995), and these effects could be different in the case of meT.

A more direct measure of *in vivo* repair is to examine the persistence of modified bases following treatment with an alkylating agent. In a strain lacking excision repair and constitutively expressing Ada, the half-life for loss of O^4-ethylthymine is similar to the half-life for loss of O^6-ethylguanine [see Figure 2 of Samson et al. (1988)]. Although it would be preferable to have data for the two methyl adducts instead, this result is at least consistent with our finding that Ada-C repairs meG and meT equally well. In the case of mammalian cells, alkylated thymines show a greater persistence than alkylated guanines (Singer, 1986). While this may indicate a low level of meT methyltransfer activity, the inability to properly control for other repair pathways makes the interpretation more difficult than in the case of *E. coli*. This is especially true since the amount of alkylating agent used is typically much higher than one would expect the cells to be subjected to under normal conditions and thus may involve other repair mechanisms to a greater extent. Establishing the relevance of *in vitro* assays by measuring substrate specificity *in vivo* will require testing several methyltransferases in a defined genetic system that controls for differences in meG/meT replication efficiency and fidelity and for differences in the specificity of other DNA repair pathways.

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