Brown, A. M., & Crothers, D. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 76*, 7387-7391.
Clore, G. M., & Gronenborn, A. M. (1982) *Biochemistry 21*, 4048-4053.
Colowick, S. P., & Womack, F. C. (1969) *Science 244*, 774-776.
de Crombrugghe, B., Busby, S., & Buc, H. (1984) *Science 224*, 831-837.
Ebright, R. H., LeGrice, S. F. J., Miller, J. P., & Krakow, J. S. (1985) *J. Mol. Biol. 182*, 91-107.
Eilen, E., & Krakow, J. S. (1977) *Biochim. Biophys. Acta 493*, 115-121.
Ferguson, R. N., Edelhoch, H., Saroff, H. A., & Robbins, J. (1975) *Biochemistry 14*, 282-289.
Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319-326.
Gronenborn, A. M., Sandulache, R., Gartner, S., & Clore, G. M. (1988) *Biochem. J. 253*, 801-807.
Heyduk, T., & Lee, J. C. (1989) *Biochemistry 28*, 6914-6924.
Heyduk, T., & Lee, J. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1744-1748.
Heyduk, E., Heyduk, T., & Lee, J. C. (1992) *J. Biol. Chem. 267*, 3200-3204.
Johnson, M. L., & Frasier, S. G. (1985) *Methods Enzymol. 117*, 301-343.
Krakow, J. S., & Pastan, I. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2529-2533.
Lee, B. J., Aiba, H., & Kyogoku, Y. (1991) *Biochemistry 30*, 9047-9054.
McKay, D. B., & Steitz, T. A. (1981) *Nature 290*, 744-749.
McKay, D. B., Weber, I. T., & Steitz, T. A. (1982) *J. Biol. Chem. 257*, 9518-9524.
Sigel, L., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346-362.
Takahashi, M., Blazy, B., & Baudras, A. (1980) *Biochemistry 19*, 5124-5130.
Tan, G.-S., Kelly, P., Rim, S, & Wartell, R. M. (1991) *Biochemistry 30*, 5076-5080.
Tsugita, A., Blazy, B., Takahashi, M., & Baudras, A. (1982) *FEBS Lett. 144*, 304-308.
Weber, I. T., & Steitz, T. A. (1987) *J. Mol. Biol. 198*, 311-326.
Wu, F. Y.-H., Nath, K., & Wu, C. W. (1974) *Biochemistry 13*, 2567-2572.
York, S. S., Lawson, R. C., & Worah, D. M. (1978) *Biochemistry 17*, 4480-4486.

Reaction of O^6-Alkylguanine-DNA Alkyltransferase with O^6-Methylguanine Analogues: Evidence That the Oxygen of O^6-Methylguanine Is Protonated by the Protein To Effect Methyl Transfer†

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ABSTRACT: The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) repairs the promutagenic O^6-methylguanine lesion by transferring the methyl group to a cysteine residue on the protein. A mechanism in which AGT activates the guanyl moiety as a leaving group by protonation of a heteroatom on guanine was probed by reacting AGT with analogues of O^6-methylguanine in which the heteroatoms were changed. The initial rates of reaction were measured at various substrate concentrations in 50 mM Hepes, 1 mM EDTA, 1 mM DTT, and 10% glycerol, pH 7.8 at 37 °C. The k_{inact} (h^{-1}) and K_{in} (mM) were determined for O^6-methylguanine (1.66 ± 0.19, 1.51 ± 0.32), 6-methoxypurine (1.07 ± 0.25, 10.6 ± 4.2), S^6-methyl-6-thioguanine (0.63 ± 0.04, 1.17 ± 0.18), 6-methylthiopurine (no reaction), Se^6-methyl-6-selenoguanine (1.76 ± 0.28, 10.6 ± 5.0), 6-methylselenopurine (2.51 ± 0.62, 15.7 ± 6.3), O^6-methyl-1-deazaguanine (1.71 ± 0.34, 14.8 ± 4.4), O^6-methyl-3-deazaguanine (1.90 ± 0.24, 2.54 ± 0.59), and O^6-methyl-7-deazaguanine (1.97 ± 0.26, 2.56 ± 0.72). These results indicate that replacement of the nitrogens does not affect the k_{inact} parameter but the K_{in} is increased upon removal of the exocyclic amino group and the nitrogen at the 1-position. Replacement of the oxygen with sulfur decreases the k_{inact}, and replacement with selenium increases the K_{in}. The results are consistent with a mechanism in which O^6-methylguanine binds to the active site of AGT with hydrogen bonds to the oxygen, the exocyclic amino group, and the nitrogen at the 1-position of the substrate. The methyl group is then displaced from the guanine as a proton is transferred to the oxygen, neutralizing the charge on the leaving group.

O^6-Alkylguanine lesions are generally believed to be important in the initiation of carcinogenesis (Pegg, 1984). Mammals and bacteria have proteins, O^6-alkylguanine-DNA alkyltransferases (EC 2.1.1.63) (AGT),[1] which repair these lesions (Pegg, 1990; Demple, 1990). AGT repairs the O^6-methylguanine lesion by transferring the methyl group from the DNA to a cysteine residue on the protein (Olsson & Lindahl, 1980; Foote et al., 1980; Demple et al., 1985; Ryberg et al., 1990). This action restores the DNA, but since the free

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[1] Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; S^6mGua, S^6-methyl-6-thioguanine; Se^6mGua, Se^6-methyl-6-selenoguanine.

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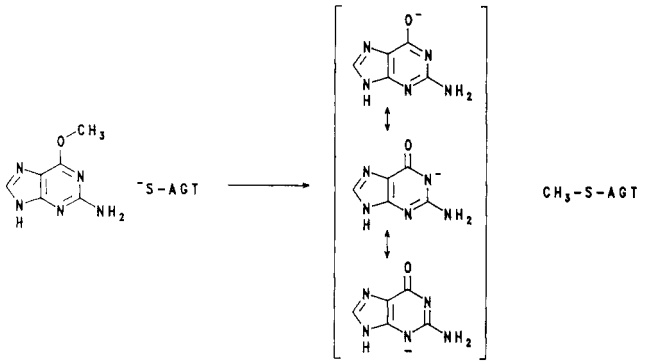
Scheme I



cysteine is not regenerated, the protein is inactivated. Two alkyltransferases have been found in *Escherichia coli*, expressed by the *ada* (Sedgwick, 1983) and *ogt* (Potter et al., 1987) genes. The 39-kDa *ada* protein (Nakabeppu et al., 1985) has two domains: the 19-kDa domain repairs O^6-alkylguanine and O^4-alkylthymine lesions (Demple et al., 1982), and the other domain repairs the alkylated phosphate backbone (McCarthy & Lindahl, 1985; Weinfeld et al., 1985) and also has a role in the regulation of DNA repair (Takano et al., 1988). The *ogt* protein is composed of one domain with the O^6-alkylguanine and O^4-alkylthymine repair activities (Margison et al., 1990). Mammalian proteins with molecular mass of about 20 kDa (Chan et al., 1990; Wilkinson et al., 1990; von Wronski et al., 1991) have been found to contain only the O^6-alkylguanine repair activity (Yarosh et al., 1985).

The native substrate for AGT is double-stranded methylated DNA. Oligonucleotides containing an O^6-methylguanine residue have also been shown to react quickly with AGT with bimolecular kinetics (Scicchitano et al., 1986; Graves et al., 1989). O^6-Methylguanine, O^6-methylguanosine, S^6-methyl-6-thioguanine (Dolan et al., 1985; Yarosh et al., 1986), and O^6-benzylguanine (Dolan et al., 1990) have been observed to react with AGT but at much reduced rates. Other alkyl chains have also been found to be removed from DNA and synthetic oligonucleotides, by the bacterial and mammalian proteins, in the general order of methyl > ethyl > propyl > butyl > isopropyl, isobutyl (Morimoto et al., 1985; Graves et al., 1990).

The mechanism by which the methyl group is transferred from the DNA to a cysteine residue on the protein is unknown. The most elementary mechanism would consist of an S_N2 attack on the methyl group by the active site cysteine acting as the nucleophile and guanine as the leaving group. Displacement of the guanyl moiety from the methyl group would result in a negative charge on the guanine, as is illustrated in Scheme I. The protein could neutralize the negative charge on the guanine and consequently make it a better leaving group by protonation of the oxygen, or the nitrogens at the 1- or 3-positions. Protonation of the exocyclic nitrogen or the nitrogen at the 7-position could also help in reducing the net charge on the leaving group through the formation of a zwitterion intermediate. In order to investigate this hypothesis, analogues of O^6-methylguanine, in which the ring nitrogens were replaced by carbon, the exocyclic nitrogen was removed, and the oxygen was replaced by sulfur and selenium, were reacted with AGT. If guanine is activated as a leaving group by proton donation, then the substrate analogue which has the proton acceptor altered should have a reduced ability to accept a proton and be activated as a leaving group.

Experimental Procedures

General. ^{1}H NMR were obtained on a Bruker AM360WB NMR with tetramethylsilane as an internal standard. Scintillation spectroscopy was performed on a Beckman LS 9800 scintillation counter using Pico-Fluor 40 (Packard) as the cocktail. Mass spectra were obtained on a Hewlett-Packard Model 5988 mass spectrometer. 6-Methoxypurine, 2-amino-6-chloropurine, 6-chloropurine, selenourea, 6-thioguanine, and 6-thiopurine were purchased from Aldrich. *N*-[^{3}H]Methyl-*N*-nitrosourea was purchased from New England Nuclear, and [^{3}H]methyl iodide was purchased from Amersham. The purity of the substrates was judged to be greater than 95% by their NMR spectra and reverse-phase HPLC chromatograms.

Syntheses. O^6-Methylguanine was synthesized by reacting sodium methoxide with 2-amino-6-chloropurine as described by Balsiger and Montgomery (1960).

S^6-Methyl-6-thioguanine (2-amino-6-methylthiopurine) and 6-methylthiopurine were synthesized by stirring vigorously 1 mmol of either 6-thioguanine or 6-thiopurine with 1.25 mmol of iodomethane in 2 mL of 0.5 N NaOH for 3 h. The suspension was filtered and washed with water, and the products were crystallized from H_2O in 90% yields. S^6-Methyl-6-thioguanine ^{1}H NMR (DMSO-d_6): 12.50 (1 H, s, NH), 7.88 (1 H, s, C8), 6.33 (2 H, s, NH_2), 2.51 (3 H, s, CH_3). 6-Methylthiopurine ^{1}H NMR (DMSO-d_6): 13.49 (1 H, s, NH), 8.69 (1 H, s, C8 or C2), 8.41 (1 H, s, C2 or C8), 2.51 (3 H, s, CH_3).

Se^6-Methyl-6-selenoguanine (2-amino-6-methylselenopurine) and 6-methylselenopurine were prepared by reacting iodomethane with the respective selenopurines, which were prepared from the reaction of selenourea and 2-amino-6-chloropurine or 6-chloropurine, respectively (Mautner, 1956). Se^6-Methyl-6-selenoguanine ^{1}H NMR (DMSO-d_6): 7.87 (1 H, s, C8), 6.31 (2 H, s, NH_2), 2.45 (3 H, s, CH_3). 6-Methylselenopurine ^{1}H NMR (DMSO-d_6): 13.51 (1 H, s, NH), 8.69 (1 H, s, C8 or C2), 8.46 (2 H, s, C2 or C8), 2.55 (3 H, s, CH_3).

2-Amino-6-[^{3}H]methylselenopurine. [^{3}H]Methyl iodide (0.5 mCi, 85 Ci/mmol, 59 nmol) in 0.5 mL of toluene was added to 2.4 mg of 2-amino-6-selenopurine (11 μmol) in 0.5 mL of 80 mM NaOH. Immediately, 12 of 10 μL of methyl iodide in 2 mL of toluene (0.8 μmol) was added, and the reaction mixture was stirred vigorously for 60 min. The mixture was neutralized with 40 μL of 1 N HCl and evaporated in vacuo. The residue was dissolved in 0.25 mL of methanol, applied to a silica gel column (10 × 1 cm), and eluted with 10% methanol/ethyl acetate. The radioactivity in the eluant was monitored by scintillation counting, and the radioactive fractions were combined to give the product in 55% yield.

2-Amino-6-[^{3}H]methylthiopurine was prepared as described above in 65% radioactive yield. The identities of the radiolabeled products were confirmed by cochromatography of the tritiated compounds with synthetic standards on silica gel TLC.

5-Amino-7-methoxy-3H-imidazo[4,5-b]pyridine (O^6-methyl-1-deazaguanine) was synthesized as described by Markees and Kidder (1956) and Gorton and Shive (1957).

6-Amino-4-methoxy-1H-imidazo[4,5-c]pyridine (O^6-Methyl-3-deazaguanine). 3-Deazaguanine (275 mg, 1.85 mmol), prepared by the method of Revankaar et al. (1984), was heated to reflux in 40 mL of trifluoroacetic anhydride for 1 h. The anhydride was removed by evaporation, and the N^2-(trifluoroacetyl)-3-deazaguanine was dissolved in 25 mL of anhydrous THF and evaporated three times to reduce the amount of trifluoroacetic acid present. PPh_3 (1.88 g, 7.0 mmol), methanol (290 μL, 7.0 mmol), and then diethyl azodicarboxylate (1.10 mL, 7.0 mmol) were added, and the mixture was stirred for 2 h at room temperature. O^6-

Methyl-N^2-(trifluoroacetyl)-3-deazaguanine was purified by silica gel chromatography by elution with 10% methanol in ethyl acetate in 70% yield. ^{1}H NMR (CD_3OD): 8.13 (1 H, s, C2), 6.79 (1 H, s, C7), 4.12 (3 H, s, methoxy). CI MS: m/z (relative intensity) 279 (5), 261 (35, M + 1), 115 (98), 114 ($CF_3CONH_3^+$), 71 (80).

The trifluoroacetyl group was removed by refluxing 100 mg of O^6-methyl-N^2-(trifluoroacetyl)-3-deazaguanine (0.38 mmol) in 100 mL of 1 N sodium methoxide in methanol for 48 h. O^6-Methyl-3-deazaguanine was purified by reverse-phase HPLC in 85% yield. A linear gradient of 0–40% methanol in 20 mM sodium phosphate, pH 6.8, at 3 mL/min on a 4.9 mm × 50 cm Whatman Partisil 10 ODS-3 column gave retention times of 30 min for O^6-methyl-3-deazaguanine and 40 min for O^6-methyl-N^2-(trifluoroacetyl)-3-deazaguanine. ^{1}H NMR (CD_3OD): 7.88 (1 H, s, C2), 5.76 (1 H, s, C7), 4.02 (3 H, s, methoxy). CI MS: m/z (relative intensity) 166 (80), 165 (100, M + 1), 164 (80).

2-Amino-4-methoxypyrrolo[2,3-d]pyrimidine (O^6-Methyl-7-deazaguanine). 7-Deazaguanine (0.56 g, 3.7 mmol), prepared as described by Davoll (1960), was converted into 2-amino-4-chloropyrrolo[2,3-*d*]pyrimidine and then 2-amino-4-methoxypyrrolo[2,3-*d*]pyrimidine in a procedure published by Seela et al. (1983). ^{1}H NMR (DMSO-d_6): 11.02 (1 H, s, NH), 6.82 (1 H, dd, C6), 6.18 (1 H, dd, C5), 5.98 (2 H, s, NH_2), 3.91 (3 H, s, methoxy).

O^6-Alkylguanine–DNA Alkyltransferase Assay. The [^{3}H]DNA substrate was prepared similarly to that described by Karran et al. (1979). Calf thymus DNA (25 mg) was reacted with 1 mCi of *N*-[^{3}H]methyl-*N*-nitrosourea (500 Ci/mol) in 5 mL of 50 mM sodium cacodylate and 1 mM EDTA, pH 6.0, followed by incubation at 80 °C for 16 h at pH 7. The DNA was then precipitated by the addition of 2 volumes of ethanol. After being washed with ethanol, the DNA was dissolved in 15 mL of 20 mM Tris-HCl and 1 mM EDTA, pH 7.8, and then dialyzed at 4 °C against two changes of 1 L of the same buffer. The activity of the enzyme was measured as described by Yarosh et al. (1986) in which the protein, which was methylated by the [^{3}H]methyl-DNA, was precipitated by acid and counted.

Purification of O^6-Alkylguanine–DNA Alkyltransferase. The homogenate was prepared by sonication of 12 g of *E. coli* BS21 (Demple et al., 1982) for 5 min (15-s sonication, 15 s on ice) in 30 mL of 50 mM Tris-HCl, 5 mM EDTA, 5 mM β-mercaptoethanol, and 10% glycerol, pH 8.3 at 4 °C. Phenylmethanesulfonyl fluoride (1 mg/mL) was added, and the homogenate was stirred for 1 h at 4 °C. The suspension was centrifuged, and the pellet was washed with 10 mL of buffer and centrifuged. The supernatant was stirred with an equal volume of DEAE-cellulose which had been equilibrated with buffer for 1 h at 4 °C. The suspension was poured into a 2.5 cm diameter column and eluted with buffer until the absorbance at 280 nm decreased to zero. The column was then eluted with buffer containing 0.5 M NaCl, and 5-mL fractions were collected. The fractions with a UV absorbance at 280 nm were collected and dialyzed overnight against 4 L of 20 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 10% glycerol, pH 8.0 at 4 °C.

The dialyzate was applied to a 2.5 × 5 cm column of double-stranded DNA–cellulose at 0.5 mL/min which had been equilibrated with 20 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 10% glycerol, pH 8 at 4 °C. The column was washed with buffer at 1 mL/min, collecting 5-mL fractions, until the absorbance of the eluant at 280 nm decreased to zero. The protein was eluted off the column with buffer containing 0.5 M NaCl.

The active fractions were concentrated to 100 μL with an Amicon Centriprep-30 and then a Centricon-10 concentrator and applied to a 30-cm Superose 12 column which was eluted with 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, and 1 mM DTT, pH 8.0, at 0.40 mL/min at 4 °C. Active fractions were combined, concentrated to 1 mL, and then dialyzed against 1 L of 20 mM Tris-HCl, 1 mM β-mercaptoethanol, and 0.1 mM EDTA, pH 7.8.

The protein was then applied to a Mono Q column (0.5 × 5 cm) equilibrated with 20 mM Tris and 1 mM DTT, pH 7.8 at 4 °C, at 1 mL/min. A 20-min linear gradient to 0.5 M NaCl was run. The active fractions were combined, diluted to 50% with glycerol, and stored at −20 °C. The protein was then analyzed by SDS–PAGE (15%) (Laemmli, 1970) and stained with silver (Meril et al., 1981).

Enzyme–Substrate Reaction. The reactions between the AGT and O^6-methylguanine analogues were initiated by the addition of 10–30 μL of enzyme solution (approximately 1.5 pmol) to 1.0 mL of 50 mM Hepes, 1.0 mM DTT, 1.0 mM EDTA, and 10% glycerol, pH 7.8 at 37 °C, containing the appropriate concentration of substrate (0–35 mM). The solutions containing the sulfur and selenium substrate analogues were made immediately prior to use to minimize the hydrolysis of the substrate. The reaction was incubated from 20 to 90 min and then quenched by the addition of DNA–substrate containing approximately 3 pmol of the radiolabeled O^6-methylguanine. The solution was then incubated for 60 min at 37 °C, during which time any remaining active AGT was [^{3}H]methylated. Subsequently, 10 μL of BSA (40 mg/mL) and then 200 μL of 50% TCA were added with shaking, and the suspension was heated at 80 °C for 30 min, cooled on ice, and filtered through a CF/C glass fiber filter. The precipitate was washed with cold 5% TCA, the precipitate and filter were transferred to a scintillation vial, and the radioactivity was measured with scintillation cocktail. This procedure was performed on samples which contained substrate and AGT and on control reactions in which no AGT was added to the solution.

Reaction of 2-Amino-6-[^{3}H]methylselenopurine and 2-Amino-6-[^{3}H]methylthiopurine with AGT. A solution of either 10 mM 2-amino-6-[^{3}H]methylselenopurine or 1.0 mM 2-amino-6-[^{3}H]methylthiopurine and either 0, 1.8, or 3.6 nM AGT in 50 mM Hepes, 1 mM EDTA, 1 mM DTT, and 20% glycerol, pH 7.8, was incubated for 2 h at 37 °C. The solutions were stored at 4 °C until they were chromatographed on Sephadex G-15 (30 × 1.5 cm). The column was eluted with 50 mM sodium phosphate, pH 7.0, at 1 mL/min, and 1-mL fractions were collected. The fractions were analyzed by counting a 200-μL aliquot with 5 mL of scintillation fluid in the scintillation counter. The fractions in the initial radioactive peak, which eluted in the void volume, were combined and dialyzed for 12 h against 2 L of 20 mM Tris-HCl and 8 M urea, pH 7.0 at 4 °C, and then against 2 L of 20 mM Tris-HCl, pH 7.0 at 4 °C. The radioactivity in the samples before and after dialysis was measured by scintillation counting.

Results

The syntheses of O^6-methylguanine (Basinger & Montgomery, 1960), Se^6-methyl-6-selenoguanine, 6-methylselenopurine (Mautner, 1956), and O^6-methyl-1-deazaguanine (Markees & Kidder, 1956; Gorton & Shive, 1957) were accomplished by published procedures. S^6-Methyl-6-thioguanine and 6-methylthiopurine were prepared from the respective thiopurine by methylation with iodomethane. O^6-Methyl-7-deazaguanine was prepared from 7-deazaguanine by reaction

Table I: Purification of O^6-Alkylguanine–DNA Alkyltransferase

procedure	AGT (pmol)	protein[a] (μg)	sp act. (pmol/mg)
homogenate	2600	56000	46
DEAE-cellulose	2500	40000	63
DNA–cellulose	1800	3700	486
Superose 12	1000	920	1087
Mono Q	750	120	6250

[a] Determined by the method of Spector (1978).

with $POCl_3$ to form the chloride, followed by reaction of sodium methoxide in methanol (Seela et al., 1983). O^6-Methyl-3-deazaguanine was prepared from 3-deazaguanine via the Mitsunobu reaction (Mitsunobu, 1981). The exocyclic amino group was protected as its trifluoroacetamide by heating to reflux 3-deazaguanine in trifluoroacetic anhydride. The protected base was then reacted with PPh_3, methanol, and diethyl azodicarboxylate in anhydrous THF at room temperature. O^6-Methyl-3-deazaguanine was purified by reverse-phase HPLC after deprotection of the exocyclic amino group in sodium methoxide in refluxing methanol. Attempts to synthesize this compound by activating the 6-position to methoxide attack by trifluoroacetic anhydride/pyridine (Fahti et al., 1990) and aromatic sulfonyl chloride/trimethylamine (Gaffney & Jones, 1982) failed.

The methyl transfer from O^6-methylguanine and analogues to AGT was monitored by measuring the concentration of active AGT at different times. The AGT–substrate reaction was quenched by the addition of an aliquot of [^{3}H]methyl-DNA. The DNA inhibits the O^6-methylguanine free base analogues from reacting with AGT, and the labeled methyl group becomes bound to the protein. The protein and DNA are precipitated by acid, and excess O^6-[^{3}H]methylguanine bound to DNA is released from the DNA into solution by heating.

AGT is not an enzyme since it does not catalyze a reaction. It does, however, have many of the properties associated with an enzyme. It is a protein, it accelerates a reaction, and it has an active site. This reaction can be envisioned as an enzyme reacting with a suicide substrate. With substrate in excess the rate equation should be pseudo first order, and it should demonstrate saturation (Walsh, 1977). The total time course of the present reactions was not done because the protein began to become inactivated after about 2 h. Therefore, the initial rates of the inactivation were determined.

The initial rate was determined according to eq 1, where t is the incubation time, dpm_t is the dpm in the precipitate after incubation with the methylated nucleobase for time t, dpm_0 is that for the control reaction in which the concentration of methylated nucleobase was zero, and dpm_c is that of the control reaction in which there was no AGT present. The initial rates of inactivation of AGT were determined for the various substrates. The data were fitted to a simple Michaelis–Menten-type kinetics (reaction 2 and eq 3) by a Marquadt nonlinear regression method (Perrella, 1988).

$$v_0/[E]_0 = [(dpm_t - dpm_0)/(dpm_c - dpm_0)](1/t) \quad (1)$$

$$E + S \rightleftarrows ES \rightarrow E_{inact} \quad (2)$$

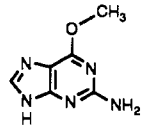
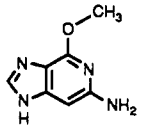
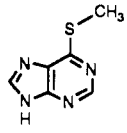
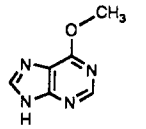
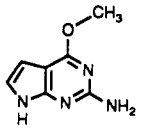
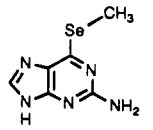
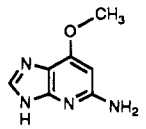
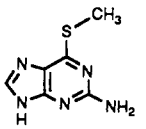
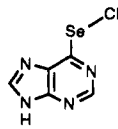
$$v_0/[E]_0 = k_{inact}[S]_0/(K_{in} + [S]_0) \quad (3)$$

AGT was purified from *E. coli* BS21 by the procedure summarized in Table I. The activity is less than that previously reported (Demple et al., 1982). SDS–PAGE with silver staining gave one band with a molecular mass of about 20 kDa, suggesting that the protein is homogeneous but contains some inactive AGT. By using the method to determine the kinetic constants as described above, the concentration of AGT does not need to be known prior to the experiment, assuming that the inactive AGT molecules do not interact with the active protein. The absolute concentration of AGT is determined for each experiment, and the $v_0/[E]_0$ is obtained. The kinetic parameters for O^6-methylguanine were obtained from several different preparations of various purities, and the k_{inact} and K_{in} were all within experimental error (data not shown).

The AGT was reacted with substrate for 20–90 min during which time up to 30% of AGT was inactivated. The initial rate values obtained were the average of three experiments. The plots of $v_0/[E]_0$ versus $[S]_0$ for O^6-methylguanine, S^6-methyl-6-thioguanine, and O^6-methyl-3-deazaguanine are presented in Figure 1. As is illustrated in Figure 1, the methyl transfer exhibits saturation, and the data are consistent with a simple Michaelis–Menten reaction scheme.

The kinetic parameters along with their standard errors for the reaction with O^6-methylguanine and analogues with AGT are presented in Table II. The k_{inact} parameters for O^6-methylguanine and the O^6-methyldeazaguanine analogues are approximately the same, between 1 and 2 h^{-1}. The effect of substitution of sulfur for oxygen decreases the k_{inact} to 0.63 h^{-1} for S^6-methyl-6-thioguanine. The reaction of 6-methylthiopurine with AGT was not measurable. Substitution of selenium for sulfur results in an increase of the k_{inact} parameter

Table II: Reaction of O^6-Methylguanine Analogues with AGT[a]

	k_{inact} (h^{-1}) K_{in} (mM)		k_{inact} (h^{-1}) K_{in} (mM)		k_{inact} (h^{-1}) K_{in} (mM)
	1.66 ± 0.19 1.51 ± 0.32		1.90 ± 0.24 2.54 ± 0.59		no reaction
	1.07 ± 0.25 10.6 ± 4.2		1.97 ± 0.26 2.56 ± 0.72		1.76 ± 0.28 10.6 ± 5.0
	1.72 ± 0.10 15.1 ± 1.7		0.63 ± 0.04 1.17 ± 0.18		2.51 ± 0.62 15.7 ± 6.3

[a] 50 mM Hepes, 1.0 mM EDTA, and 1.0 mM DTT, pH 7.8 at 37 °C.

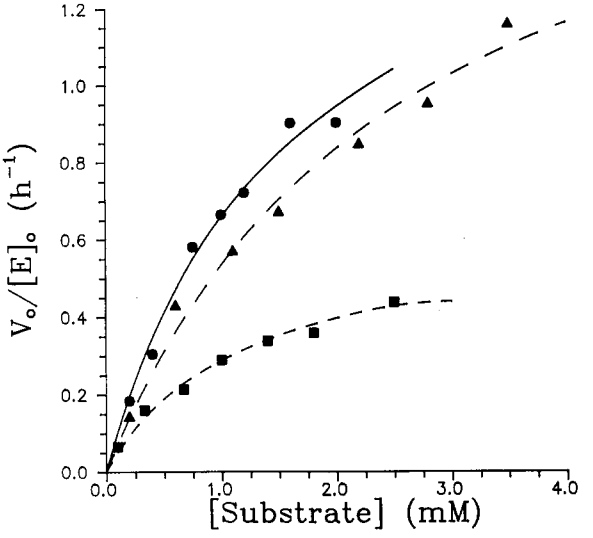


FIGURE 1: Plot of initial rate versus substrate concentration for the reaction of AGT (1–3 nM) with O^6-methylguanine (●, —), O^6-methyl-3-deazaguanine (▲, - - -), and S^6-methyl-6-thioguanine (■, – –) in 50 mM Hepes, 1 mM EDTA, 1 mM DTT, and 10% glycerol, pH 7.80 at 37 °C.

to 1.76 ± 0.28 for Se^6-methyl-6-selenoguanine and 2.51 ± 0.62 for 6-methylselenopurine.

The K_{in} parameters for O^6-methylguanine and the O^6-methyldeazaguanine analogues are between 1 and 3 mM except when the exocyclic amino group was removed and the nitrogen at the 1-position was replaced by carbon. In those analogues the K_{in} rose to 15.1 ± 1.7 mM for O^6-methyl-1-deazaguanine and 10.6 ± 4.2 mM for 6-methoxypurine. Replacement of the oxygen for selenium also resulted in a 10-fold increase in the K_{in}.

The assay employed for the determination of the methyl transfer is an indirect assay. It measures the activity of the protein. The activity of the AGT will decrease as the methyl group is transferred, but it is possible that a very tight binding of an O^6-methylguanine analogue to AGT without the transfer of the methyl group will also inhibit the protein activity. Dolan et al. (1985) showed that AGT removes the methyl group from O^6-methylguanine. To test whether this is the case for the sulfur and selenium analogues, Se^6-[^{3}H]methyl-6-selenoguanine and S^6-[^{3}H]methyl-6-thioguanine were reacted with AGT. The protein was isolated by chromatography on Sephadex G-15, and the extent to which the protein was radiolabeled was determined by scintillation spectroscopy. As presented in Table III, 18–28% of the protein is labeled. The protein was then dialyzed against 8 M urea to denature the protein to allow noncovalently bound [^{3}H]methyl groups to dialyze away from the protein. The results presented in Table III show that the radiolabel does not dialyze away from the protein, suggesting that the label is covalently bound to the protein.

Discussion

The hypothesis that AGT activates guanine as a leaving group by protonation of one of the heteroatoms on guanine was examined by reacting analogues of O^6-methylguanine in which the heteroatoms were changed. The rate of methyl transfer was determined by measuring the rate of inactivation of AGT.

This assay to determine methyl transfer is an indirect method. The rate of inactivation is measured, not the rate of methyl transfer. Although methyl transfer to the protein will inactivate it, AGT can potentially be inactivated by a very tight noncovalent complex. Dolan et al. (1985) demonstrated that O^6-methylguanine transfers the methyl group to the protein by showing that the reaction of AGT with O^6-methyl[^{3}H]-guanine liberated [^{3}H]guanine. It is assumed that the deazaguanine analogues also inactivate AGT by this method. Changing the heteroatom from oxygen to either sulfur or selenium can potentially introduce new interactions between the protein and substrate which can result in a tight binding complex between AGT and substrate.

Table III: Amount of [^{3}H]Methyl Groups Bound from the Reaction of AGT with [^{3}H]S^6mGua and [^{3}H]Se^6mGua[a]

	[^{3}H]S^6mGua[b]		[^{3}H]Se^6mGua[c]	
AGT (pmol)	Sephadex[d]	dialysis[e]	Sephadex[d]	dialysis[e]
0.0	0		0	
1.8	0.32	0.30	0.49	0.45
3.6	0.65	0.55	0.99	0.88

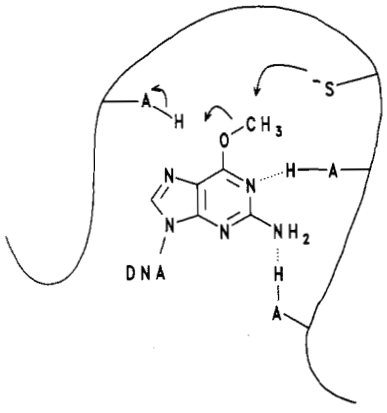
[a] Expressed in picomoles of methyl groups bound to AGT. Each reaction was carried out a single time in 1 mL of 50 mM Hepes, 1 mM EDTA, 1 mM DTT, and 10% glycerol, pH 7.8 at 37 °C. [b] 1.0 mM S^6-[^{3}H]methyl-6-thioguanine at 5.6 Ci/mmol. [c] 10 mM Se^6-[^{3}H]-methyl-6-selenoguanine at 6.2 Ci/mmol. [d] Chromatography on Sephadex G-15 (30 × 1.5 cm) and elution with 50 mM sodium phosphate, pH 7.8 at 22 °C. [e] Dialysis against 2 L of 20 mM Tris-HCl and 8 M urea, pH 7.8 at 4 °C, for 12 h and then against 2 L of 20 mM Tris-HCl, pH 7.8 at 4 °C, for 12 h.

Since it was found that the sulfur and selenium O^6-methylguanine analogues inactivated AGT, the possibility of methyl transfer was examined. AGT was reacted with the radiolabeled methyl derivatives, S^6-[^{3}H]methyl-6-thioguanine and Se^6-[^{3}H]methyl-6-selenoguanine. The protein complex was desalted with Sephadex G-15, and it was found that in the reaction with S^6-[^{3}H]methyl-6-thioguanine about 18% of the AGT molecules were labeled. The reaction of Se^6-[^{3}H]-methyl-6-selenoguanine with AGT resulted in the labeling of about 25% of the protein molecules. Dialysis under denaturing conditions did not release any radioactivity from the protein, indicating that the radiolabel is covalently bound to the protein. These results indicate that S^6-[^{3}H]methyl-6-thioguanine and Se^6-[^{3}H]methyl-6-selenoguanine both inactivate AGT by transfer of the methyl group to the protein.

The reactions between O^6-methylguanine and analogues with AGT all exhibited simple Michaelis–Menten kinetics. Replacement of the nitrogens at the 3- and 7-positions with carbon did not significantly affect the rate of methyl transfer, indicating that these positions are not directly involved in the reaction. Replacement of the nitrogen at the 1-position and removal of the exocyclic amino group resulted in a decrease of the rate of reaction through an increase in the K_{in}. If any of the nitrogens were an essential proton acceptor for the methyl transfer, then the k_{inact} parameter would be significantly reduced for that O^6-methyldeazaguanine substrate when compared with the other substrates. Since the k_{inact} was not significantly affected by the nitrogen substitutions, these positions do not appear to be involved in a proton donation which is essential for the methyl transfer to occur. If it is assumed that K_{in} measures the dissociation of the substrate from the protein ($K_{in} = K_S$), then it can be concluded from the larger K_{in}'s for O^6-methyl-1-deazaguanine and 6-methoxypurine that these positions are involved in the binding of the substrate to the protein.

Analysis of the oxygen substitutions is not as straightforward as the nitrogen substitutions because there are two competing trends. First, the substitution of sulfur and selenium for oxygen would be expected to decrease the efficiency of the proton donation from AGT to the heteroatom. Sulfur and selenium are larger than oxygen. If AGT were optimized to donate a

Scheme II



proton to the oxygen of O^6-methylguanine, then the distances in the active site would be different for the sulfur and selenium substrate analogues. Therefore, proton donation to the sulfur and selenium substrate analogues would not be as efficient as for the native substrate. If proton donation were part of the rate-determining step, then the rate of methyl transfer should decrease in the order O^6-methylguanine > S^6-methyl-6-thioguanine > Se^6-methyl-6-selenoguanine.

Second, the selenium and sulfur analogues are more acidic than O^6-methylguanine. O^6-Methylguanine has a pK_a of 9.2; S^6-methyl-6-thioguanine, 8.2; and Se^6-methyl-6-selenoguanine, 7.7 (Chu, 1971). This indicates that the selenium and sulfur guanine analogues should be better leaving groups, and consequently, the k_{inact} parameter should increase in the order O^6-methylguanine < S^6-methyl-6-thioguanine < Se^6-methyl-6-selenoguanine.

Both trends can be visualized in the results. The change of the oxygen on O^6-methylguanine to sulfur decreases the k_{inact}, and the subsequent change to selenium increases the k_{inact}. These results are consistent with proton donation to oxygen in which replacement of the oxygen with sulfur and selenium decreases the proton donation ability. Consequently, the k_{inact} for S^6-methyl-6-thioguanine is reduced from that of O^6-methylguanine. However, with the insertion of selenium, the leaving group is good enough such that proton donation is not needed for catalysis, and consequently, the k_{inact} for Se^6-methyl-6-selenoguanine is larger than that for S^6-methyl-6-thioguanine.

The results are consistent with the mechanism illustrated in Scheme II. O^6-Methylguanine binds to the active site with hydrogen bonds to the oxygen, the exocyclic amino group, and the nitrogen at the 1-position. The hydrogen bond to the N^2 group is shown with the amino group being the hydrogen acceptor. There is no experimental evidence as to whether the exocyclic amino group is donating or accepting a hydrogen bond. However, if O^4-methylthymine is repaired in a similar mechanism at this active site, then the O^2-position of O^4-methylthymine would occupy the same position as the N^2-position of O^6-methylguanine. Since the oxygen of thymine can only be a hydrogen-bond acceptor, it seems likely that the exocyclic amino group of O^6-methylguanine also accepts a hydrogen bond from the protein. The cysteine then displaces the methyl group from the guanyl moiety in a direct nucleophilic attack as a proton is transferred to the oxygen neutralizing the charge on the leaving group.

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References

Balsiger, R. W., & Montgomery, J. A. (1960) *J. Org. Chem. 25*, 1573–1575.

Bhattacharyya, D., Tano, K., Bunick, G. J., Uberbacher, E. C., Behnke, W. D., & Mitra, S. (1988) *Nucleic Acids Res. 16*, 6397–6410.

Chan, C. L., Wu, Z., Eastman, A., & Bresnick, E. (1990) *Carcinogenesis 11*, 1217–1221.

Chu, S.-H. (1971) *J. Med. Chem. 14*, 254–255.

Cook, P. D., Rousseau, R. J., Mian, A. M., Meyer, R. B., Jr., Dea, P., Ivanovics, G., Streeter, D. G., Witkowski, J. T., Stout, M. G., Simon, L. N., Sidwell, R. W., & Robins, R. K. (1975) *J. Am. Chem. Soc. 97*, 2916–2917.

Cook, P. D., Rousseau, R. J., Mian, A. M., Dea, P., Meyer, R. B., Jr., & Robins, R. K. (1976) *J. Am. Chem. Soc. 98*, 1492–1498.

Davoll, J. (1960) *J. Chem. Soc.*, 131–138.

Demple, B. (1990) in *Protein Methylation* (Paik, W. M., Kim, S., Eds.) pp 285–304, CRC Press, Inc., Boca Raton, FL.

Demple, B., Jacobsson, A., Olsson, M., Robins, P., & Lindahl, T. (1982) *J. Biol. Chem. 257*, 13776–13780.

Demple, B., Sedgwick, B., Totty, N., Waterfield, M. D., & Lindahl, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2688–2692.

Dolan, M. E., Morimoto, K., & Pegg, A. E. (1985) *Cancer Res. 45*, 6413–6417.

Dolan, M. E., Moschel, R. C., & Pegg, A. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5368–5372.

Fahti, R., Goswami, B., Kung, P.-P., Gaffney, B., & Jones, R. A. (1990) *Tetrahedron Lett. 31*, 319–320.

Foote, R. S., Mitra, S., & Pal, B. C. (1980) *Biochem. Biophys. Res. Commun. 97*, 654–659.

Gaffney, B. L., & Jones, R. A. (1982) *Tetrahedron Lett. 23*, 2253–2256.

Gorton, B. S., & Shive, W. (1957) *J. Am. Chem. Soc. 79*, 670–672.

Graves, R. J., Li, B. F. L., & Swann, P. F. (1989) *Carcinogenesis 10*, 661–666.

Hora, J. F., Eastman, A., & Bresnick, E. (1983) *Biochemistry 22*, 3759–3763.

Karran, P., Lindahl, T., & Griffin, B. (1979) *Nature 280*, 76–77.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lindahl, T. (1982) *Annu. Rev. Biochem. 51*, 61–87.

Margison, G. P., Cooper, D. P., & Potter, P. M. (1990) *Mutat. Res. 233*, 15–21.

Markees, D. G., & Kidder, G. W. (1956) *J. Am. Chem. Soc. 78*, 4130–4135.

Mautner, H. G. (1956) *J. Am. Chem. Soc. 78*, 5292–5294.

McCarthy, T. V., & Lindahl, T. (1985) *Nucleic Acids Res. 13*, 2683–2698.

Merril, C. R., Goldman, D., Sedman, S. A., & Ebert, M. H. (1981) *Science 211*, 1437–1438.

Mitsunobu, O. (1981) *Synthesis 1*, 1981.

Montgomery, J. A., & Wood, N. F. (1964) *J. Org. Chem. 29*, 734–738.

Morimoto, K., Dolan, M. E., Scicchitano, D., & Pegg, A. E. (1985) *Carcinogenesis 6*, 1027–1031.

Myers, G. M., & Sancar, A. (1989) *Chem. Res. Toxicol. 2*, 197–226.

Nakabeppu, Y., Kondo, H., Kawabata, S., Iwanaga, S., & Sekiguchi, M. (1985) *J. Biol. Chem. 260*, 7281–7288.

Olsson, M., & Lindahl, T. (1980) *J. Biol. Chem. 255*, 10569–10571.

Pegg, A. E. (1984) *Cancer Invest. 2*, 223-231.
Pegg, A. E. (1990) *Cancer Res. 50*, 6119-6129.
Pegg, A. E., Wiest, L., Foote, R. S., Mitra, S., & Perry, W. (1983) *J. Biol. Chem. 258*, 2327-2333.
Pegg, A. E., Scicchitano, D., & Dolan, M. E. (1984) *Cancer Res. 44*, 3806-3811.
Perrella, F. W. (1988) *Anal. Biochem. 174*, 437-447.
Potter, P. M., Wilkinson, M. C., Fitton, J., Brennand, J., Cooper, D. P., & Margison, G. P. (1987) *Nucleic Acids Res. 15*, 9177-9193.
Rebeck, G. W., Coons, S., Carroll, P., & Samson, L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3039-3043.
Revankar, G. R., Gupta, P. K., Adams, A. D., Dalley, N. K., McKernan, P. A., Cook, P. D., Canonico, P. G., & Robins, R. K. (1984) *J. Med. Chem. 27*, 1389-1396.
Robins, R. K., Horner, J. K., Greco, C. V., Noell, C. W., & Beames, C. G., Jr. (1963) *J. Org. Chem. 28*, 3041-3046.
Ryberg, B., Hall, J., & Karran, P. (1990) *Nucleic Acids Res. 18*, 17-21.
Scicchitano, D., & Pegg, A. E. (1982) *Biochem. Biophys. Res. Commun. 109*, 995-1001.
Scicchitano, D., Jones, R. A., Kuzmich, S., Gaffney, B., Lasko, D. D., Essigmann, J. M., & Pegg, A. E. (1986) *Carcinogenesis 7*, 1383-1386.
Sedgwick, B. (1983) *Mol. Gen. Genet. 191*, 466-472.
Sedgwick, B., Robins, P., Totty, N., & Lindahl, T. (1988) *J. Biol. Chem. 263*, 4430-4433.
Seela, F., Kehne, A., & Winkeler, H.-D. (1983) *Liebigs Ann. Chem.*, 137-146.
Smith, R. H., Jr., Koepke, S. R., Tondeur, Y., Denlinger, C. L., & Michejda, C. J. (1985) *J. Chem. Soc., Chem. Commun.*, 936-937.
Souliotis, V. L., Giannopoulos, A., Koufakis, I., Kaila, S., Dimopoulos, C., & Kyrtopoulos, S. A. (1989) *Carcinogenesis 10*, 1203-1208.
Spector, T. (1978) *Anal. Biochem. 86*, 248-254.
Takano, K., Nakabeppu, Y., & Sekiguchi, M. (1988) *J. Mol. Biol. 201*, 261-271.
Tano, K., Bharracharyya, D., Foote, R. S., Mural, R. J., & Mitra, S. (1988) *J. Bacteriol. 171*, 1535-1543.
Temple, C., Jr., Smith, B. H., Kussner, C. L., & Montgomery, J. A. (1976) *J. Org. Chem. 41*, 3784-3788.
von Wronski, M. A., Shiota, S., Tano, K., Mitra, S., Bigner, D. D., & Brent, T. P. (1991) *J. Biol. Chem. 266*, 1064-1070.
Walsh, C. T. (1977) *Horiz. Biochem. Biophys. 3*, 36-81.
Weinfeld, M., Drake, A. F., Saunders, J. K., & Paterson, M. C. (1985) *Nucleic Acids Res. 13*, 7067-7077.
Wilkinson, M. C., Potter, P. M., Cawkwell, L., Georgradis, P., Patel, D., Swann, P. F., & Margison, G. P. (1989) *Nucleic Acids Res. 17*, 8475-8483.
Wilkinson, M. C., Cooper, D. P., Southan, C., Potter, P. M., & Margison, G. P. (1990) *Nucleic Acids Res. 18*, 13-16.
Yarosh, D. B. (1985) *Mutat. Res. 145*, 1-16.
Yarosh, D. B., Fornace, A. J., & Day, R. S., III (1985) *Carcinogenesis 6*, 949-953.
Yarosh, D. B., Hurst-Calderone, S., Babich, M. A., & Day, R. S., III (1986) *Cancer Res. 46*, 1663-1668.

Excision Repair of DNA in Nuclear Extracts from the Yeast *Saccharomyces cerevisiae*†

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ABSTRACT: Excision repair of DNA is an important cellular response to DNA damage caused by a broad spectrum of physical and chemical agents. We have established a cell-free system in which damage-specific DNA repair synthesis can be demonstrated in vitro with nuclear extracts from the yeast *Saccharomyces cerevisiae*. Repair synthesis of UV-irradiated plasmid DNA was observed in a radiation dose-dependent manner and was unaffected by mutations in the *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, or *APN1* genes. DNA damaged with *cis*-platin was not recognized as a substrate for repair synthesis. Further examination of the repair synthesis observed with UV-irradiated DNA revealed that it is dependent on the presence of endonuclease III-sensitive lesions in DNA, but not pyrimidine dimers. These observations suggest that the repair synthesis observed in yeast nuclear extracts reflects base excision repair of DNA. Our data indicate that the patch size of this repair synthesis is at least seven nucleotides. This system is expected to facilitate the identification of specific gene products which participate in base excision repair in yeast.

Ultraviolet (UV) light, ionizing radiation, and a variety of chemical agents can cause chemical alterations in the nitrogenous bases of DNA (Friedberg, 1985). Base excision repair and nucleotide excision repair are two important cellular responses to such genetic insults. Base excision repair is initiated by the specific recognition of a variety of chemically altered or inappropriate (such as uracil or hypoxanthine) bases by DNA glycosylases. These enzymes catalyze the hydrolysis of the *N*-glycosyl bond linking the bases to the sugar-phosphate backbone, resulting in their excision as free bases. The resulting sites of base loss are then attacked by an apurinic/

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