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Formation of a covalent complex between methylguanine methyltransferase and DNA via disulfide bond formation between the active site cysteine and a thiol-containing analog of guanine

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ABSTRACT

DNA repair methyltransferases (MTases) remove methyl or other alkyl groups from the O^6 position of guanine or the O^4 position of thymine by transfering the group to an active site cysteine. In order to trap an MTase–DNA complex via a disulfide bond, 2′-deoxy-6-(cystamine)-2-aminopurine (d^6Cys^2AP) was synthesized and incorporated into oligonucleotides. d^6Cys^2AP has a disulfide bond within an alkyl chain linked to the 6 position of 2,6-diaminopurine, which disulfide can be reduced to form a free thiol. Addition of human MTase to reduced oligonucleotide resulted in a protein–DNA complex that was insensitive to denaturation by SDS and high salt, but which readily dissociated in the presence of dithiothreitol. Formation of this complex was prevented by methylation of the active site cysteine. Evidence that the active site cysteine is directly involved in disulfide bond formation was obtained by N-terminal sequencing of peptides that remained associated with DNA after proteolysis of the complex.

INTRODUCTION

DNA methylguanine methyltransferases (MTases) repair alkylation damage at the O^6 position of guanine and at the O^4 position of thymine (1,2). MTases can repair a variety of premutagenic lesions arising from exposure of DNA to alkylating agents, including methyl, ethyl and benzyl groups (1,3). The modifed bases are repaired in a suicide reaction by transfering the alkyl group to an active site cysteine, forming an irreversible thioether linkage (4,5). Thus, MTases are not true enzymes.

The structure of the MTase domain of the *Escherichia coli* homolog Ada has been determined in the absence of DNA (6). In this structure, the active site cysteine is substantially buried, suggesting that a conformational change must occur on binding of substrate or during the repair process. Circular dichroism has provided some evidence for a conformational change upon DNA binding, but there is no detailed structural information regarding the nature of this change (7). A stable MTase–DNA complex would allow further biochemical and structural characterization of this unique DNA repair protein.

It has proven difficult to obtain stable and discrete MTase–DNA complexes. There are no ligands or co-factors in the reaction which might be removed or modified and addition of MTase to methylated DNA results in rapid and irreversible inactivation of the protein. Furthermore, we have been unable to detect specific binding by variants of human MTase in which the active site cysteine was mutated to serine or alanine (J.Deng and N.D.Clarke, unpublished results).

The approach we describe here is to design a thiol-containing oligonucleotide that will form a crosslink with the active site cysteine. The ability to introduce reactive thiols into synthetic oligonucleotides is a useful biochemical tool. For example, disulfide bond formation has been used to trap conformationally stressed nucleic acid structures (8,9) and map RNA structure (10) and it could facilitate protein–DNA interactions in which strand separation or other helical distortions are required (11). Commercially available supports and phosphoramidites that contain disulfides or other protected sulfhydryl groups provide convenient mechanisms for efficient conjugation of oligonucleotides (12). We have employed synthetic strategies similar to those used above to produce a thiol-containing synthetic oligonucleotide capable of crosslinking to the active site cysteine of the human MTase through disulfide bond formation.

A phosphoramidite was prepared in which the N^6 of 2,6-diaminopurine (DAP) was derivatized with a disulfide-containing alkyl linker. Incorporation into an oligonucleotide and subsequent reduction of the disulfide leaves a reactive thiol positioned on a two carbon tether in the major groove of the DNA. This thiol reacts with the active site cysteine of MTase to form a covalent disulfide bond. The complex is resistant to high salt and denaturing conditions and may be useful for further biochemical characterization of this unusual DNA repair protein. However, the crosslinking efficiency is not yet high enough for structural studies.

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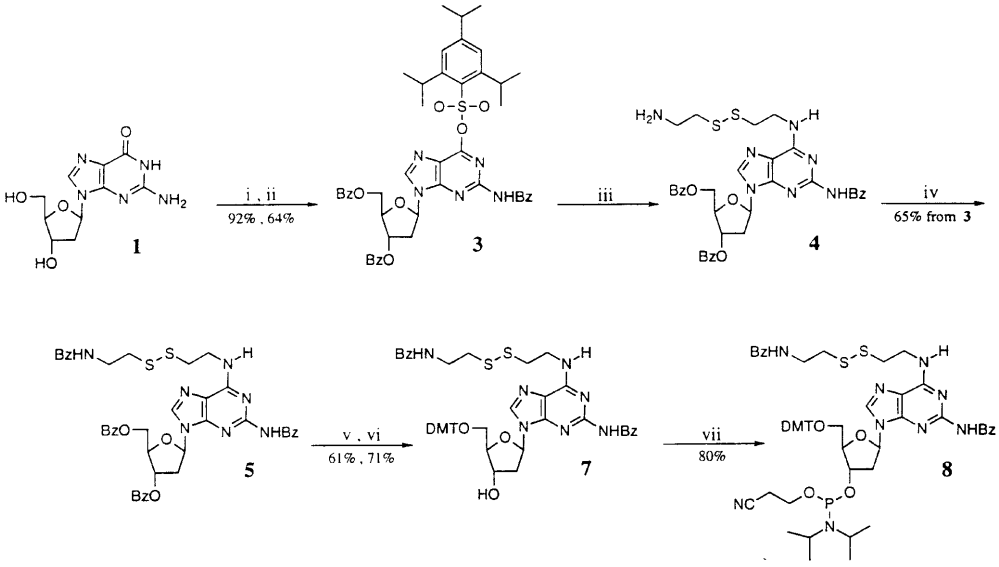


Figure 1. Synthesis of the dimethoxytrityl (DMT)-protected phosphoramidite of d^{6}Cys^{2}AP: (i) benzoic anhydride, pyridine, reflux 12 h; (ii) 2,4,6-triisopropylbezene-sulphonyl chloride, *N*,*N*-diisopropylethylamine, CH_2Cl_2, 1 h; (iii) cystamine dihydrochloride, DBU, pyridine, 1 h; (iv) benzoic anhydride, pyridine, room temperature, 12 h; (v) NaOH, MeOH, pyridine, –20°C, 30 min; (vi) DMTCl, pyridine, 1.5 h; (vii) *N*,*N*-diisopropylammonium tetrazolide, bis-(diisopropylamino)-*b*-cyanoethoxy phosphine, CH_2Cl_2, 2.5 h.

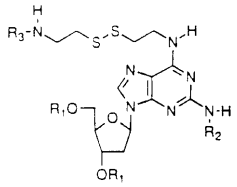
MATERIALS AND METHODS

General methods

2′-Deoxyguanosine was purchased from US Biochemical (Cleveland, OH). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and anhydrous solvents were used as is. TLC was conducted on EM plastic-backed sheets (silica gel 60 F254, 0.2 mm). Flash column chromatography was performed on Merck silica gel 60, 230–400 mesh. Absorption spectra were recorded by a Varian DMS100S UV/Vis spectrophotometer. HPLC separations and analyses were conducted on a Varian 9010/50 system. ^{1}H and ^{31}P NMR were performed on a Bruker AMX 300 spectrophotometer; chemical shifts are in p.p.m. and proton chemical shifts are reported relative to internal tetramethylsilane ($CDCl_3$) or to residual solvent peaks (DMSO); ^{31}P spectra were collected with broad band proton noise decoupling and referenced to an external 85% H_3PO_4 standard. High resolution mass spectral analyses (HRMS), MALDI, were performed by the Middle Atlantic Mass Spectrometry Laboratory, an NSF Regional Instrumentaion Facility at Johns Hopkins University. N-Terminal sequence analysis was conducted by the core facility in the Department of Biological Chemistry at Johns Hopkins University. The enzymes shrimp alkaline phosphatase (SAP) and T4 polynucleotide kinase (PNK) were purchased from US Biochemicals or New England Biolabs; snake venom phosphodiesterase (SVP) from *Crotalus durissus* was purchased from Boehringer Mannheim (Indianapolis, IN). The human MTase was a gift from Dr Phil Potter and was dialyzed thoroughly against TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) before use.

Chemical synthesis of a d^{6}Cys^{2}AP phosphoramidite

The overall scheme for synthesis is shown in Figure 1; compound **6** and its deprotection products are shown in Figure 2.



6 R_1=H; R_2=Bz; R_3=Bz
6a R_1=H; R_2=H; R_3=H
6b R_1=H; R_2=Bz; R_3=H
6c R_1=H; R_2=H; R_3=Bz

Figure 2. Structure of 2′-deoxy-6-(cystamine)-2-aminopurine (d^{6}Cys^2–AP) nucleoside [**6a**] and partially deprotected variants.

3′,5′,N^2-Tribenzoyl-2′-deoxy-6-(cystamine)-2-aminopurine [4]. Compound **3** (2.65 g, 4.6 mmol), prepared as described previously (13), was dissolved in 50 ml anhydrous pyridine and the reaction vessel stirred at room temperature under a stream of dry nitrogen. To a separate reaction flask containing 35 ml anhydrous pyridine, 10 equivalents cystamine dihydrochloride (10.35 g, 46 mmol) and 20 equivalents 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) (14 ml, 90 mmol) were added. Heating and agitation yielded a solution which was transferred to the reaction vessel containing **3**. The reaction was stirred at room temperature for 1 h, which resulted in the complete disappearance of **3** (R_f 0.36) and the appearance of a new spot [**4**] at the origin as monitored by TLC ($CHCl_3$/MeOH, 99:1). The mixture was added to 400 ml $CHCl_3$ and immediately extracted with saturated NaCl (5×400 ml). The organic layer was dried over Na_2SO_4 and the solvent removed by rotary evaporation (*in vacuo*). Overnight drying under vacuum resulted in an orange colored foam **4** which was used without further purification.

3′,5′,N^2,N^{cys}-Quadrabenzoyl-2′-deoxy-6-(cystamine)-2-amino-purine [5]. The foam containing **4** was dissolved in 50 ml

anhydrous pyridine to which benzoic anhydride (2.1 g, 2 equivalents **3**, 9.2 mmol) and dimethylaminopyridine (DMAP; 0.35 g) were added. The reaction was stirred at room temperature and monitored by TLC ($CHCl_3$/MeOH, 95:5). Overnight incubation resulted in complete conversion of **4** (R_f origin) to a faster moving spot **5** (R_f 0.40). The reaction was quenched by addition of 20 ml 5% $NaHCO_3$ and stirred for 1 h. The solvent was removed by rotary evaporation and co-evaporated twice with toluene. The residue was dissolved in 200 ml $CHCl_3$ and washed with dilute $NaHCO_3$ (1 × 200 ml) and with saturated NaCl (2 × 200 ml). The organic layer was dried over Na_2SO_4 and the solvent removed by rotary evaporation (*in vacuo*) to yield a foam. The foam was dissolved in 10 ml $CHCl_3$ and purified by flash chromatography on a silica gel column ($CHCl_3$/MeOH, 97.5:2.5). Fractions containing the product **5** were dried to give a yellow foam. Yield 2.46 g or 3 mmol product (65% from **3**). TLC ($CHCl_3$/MeOH, 95:5) R_f 0.40. UV (ethanol): λ_{max} = 229, 273 nm; λ_{min} = 215, 256 nm. ^{1}H-NMR (DMSO) δ = 10.39 (s,1H, NH), 8.64 (t, 1H, NH), 8.05 (d, 2H, Ar-H), 7.94 (d, 2H, Ar-H), 7.84 (m, 4H, Ar-H), 7.72–7.41 (m, 14H, NH, Ar-H,H8), 6.47 (t, 1H, H1′), 5.87 (m, 1H, H3′), 4.74–4.58 (m, 3H, H4′,5′,5′′), 3.69 (m, 2H, -N-CH2-), 3.55 (q, 2H, -N-CH2-), 3.07 (m, 2H, -S-CH2-), 3.95 (m, 2H, -S-CH2-), 3.42 (m, 1H, H2′), 2.73 (m, 1H, H2′′) p.p.m.

N^2,N^{cys}-Dibenzoyl-2′-deoxy-6-(cystamine)-2-aminopurine [6]. Compound **5** (2.46 g, 3 mmol) was dissolved in 40 ml pyridine. Methanol (4 ml) was added and the reaction vessel cooled to –20°C in a salt/ice bath. To the reaction vessel 2 M sodium hydroxide (3.6 ml) was added and the reaction mixture was stirred for 30 min. The reaction was monitored by following the disappearance of **5** by TLC ($CHCl_3$/MeOH, 95:5). The reaction mixture was neutralized with pyridinium dowex 50W-X8, which was removed by filtration and washed with a 1:4 water:pyridine mixture. The filtrate was concentrated by rotary evaporation (*in vacuo*) and co-evaporated twice with toluene to yielded an orange colored foam. The foam was dissolved in 10 ml $CHCl_3$ and purified by flash chromatography on a silica gel column ($CHCl_3$/MeOH, 92.5:7.5). Fractions containing **6** were dried to give a yellow foam. Yield 1.12 g or 1.84 mmol (61% yield from **5**). TLC ($CHCl_3$/MeOH, 90:10) R_f 0.17. UV (ethanol): λ_{max} = 233, 272 nm; λ_{min} = 264 nm. HRMS (MALDI) for $C_{28}H_{31}O_5N_7S_2$ (M+ H) calculated 610.72, found 610.90. ^{1}H-NMR (DMSO) δ = 10.41 (s,1H, NH), 8.63 (t, 1H, NH), 8.27 (s, 1H, H8), 8.01 (t, 1H, NH), 7.90 (d, 2H, Ar-H), 7.82 (d, 2H, Ar-H), 7.58–7.42 (m, 6H, Ar-H), 6.29 (t, 1H, H1′), 5.27 (d, 0.5H, 3′-OH), 4.93 (t, 0.5H, 5′-OH), 4.37 (m, 1H, H4′), 3.84 (q, 1H, H3′), 3.67 (m, 2H, H5′), 3.61–3.46 [m, 4H, 2×(-N-CH2-)], 3.06 (t, 2H, -S-CH2-), 2.94 (t, 2H, -S-CH2-), 2.23 (m, 1H, H2′′) p.p.m.

5′-O-(4,4′-Dimethoxytrityl)-N^2,N^{cys}-dibenzoyl-2′-deoxy-6-(cystamine)-2-aminopurine [7]. Compound **6** (1.12 g, 1.84 mmol) was dissolved in 20 ml anhydrous pyridine to which 1.5 equivalents 4,4′-dimethoxytritylchloride (0.94 g, 2.77 mmol) were added. The reaction was stirred at room temperature and monitored by TLC ($CHCl_3$/MeOH, 95:5). Complete conversion of **6** to a faster moving product required 1.5 h, at which time the reaction was quenched by addition of 10 ml MeOH. The solvent was removed by rotary evaporation and co-evaporated twice with toluene. The residue was dissolved in 200 ml $CHCl_3$ and washed with dilute $NaHCO_3$ (2 × 200 ml) and with saturated NaCl (1 × 200 ml). The organic layer was dried over Na_2SO_4 and the solvent removed by rotary evaporation (*in vacuo*) to yield a light brown oil. The oil was dissolved in 10 ml $CHCl_3$ and purified by flash column chromatography on a silica gel column ($CHCl_3$/MeOH/TEA, 94:4:2). A second column purification step was included using ($CHCl_3$/MeOH/TEA, 96:2:2) to yield pure product. Fractions containing **7** were dried by rotary evaporation (*in vacuo*) to give a pale yellow foam. Yield 1.18 g or 1.30 mmol (71% yield from **6**). TLC ($CHCl_3$/MeOH/TEA, 96:2:2) R_f 0.36. UV (ethanol): λ_{max} = 202, 230, 272 nm; λ_{min} = 220, 260 nm. HRMS (MALDI) for C28H31O5N7S2 (M+ H) calculated 913.096, found 913.40. ^{1}H-NMR ($CDCl_3$) δ = 8.65 (t,1H, NH), 8.35 (t, 1H, NH), 7.89–7.84 (m, 2H, H8,NH), 7.78 (d, 2H, Ar-H), 7.74 (d, 2H, Ar-H), 7.60–6.71 (m, 13H, Ar-H), 6.46 (t, 1H, H1′), 6.29 (t, 1H, H1′), 4.81 (m, 1H, H4′), 4.04 (m, 1H, H3′), 3.76 (s, 6H, -O-CH3), 3.50–3.35 [m, 4H, 2× (-N-CH2-)], 3.09–2.98 [m, 4H, 2×(-N-CH2-)], 2.28 (m, 1H, H2′) p.p.m.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[(2-cyanoethyl)-N,N-diisopropyl-aminophosphoramidite]-N^2,N^{cys}-dibenzoyl-2′-deoxy-6-(cystamine)-2-aminopurine [8]. Compound **7** (300 mg, 0.33 mmol) was dissolved in 10 ml anhydrous CH_2Cl_2 to which 0.75 equivalents diisopropylammonium tetrazolide (42 mg, 0.25 mmol) were added. To the stirring solution at room temperature, 1.5 equivalents 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite (0.16 ml, 0.5 mmol) were added dropwise. The reaction was monitored by TLC ($CHCl_3$/MeOH/TEA, 97.5:0.5:2). The reaction was quenched by addition of 1 ml MeOH. The solvent was removed by rotory evaporation and the resulting oil dissolved in 20 ml ethyl acetate. The organic layer was washed with dilute $NaHCO_3$ (2 × 40 ml) and with saturated NaCl (1 × 40ml). The organic layer was dried over Na_2SO_4 and the solvent removed by rotory evaporation (*in vacuo*) to yield a brown foam. The brown foam was dissolved in 5 ml $CHCl_3$/MeOH/TEA (97.5:0.5:2) and purified by flash column chromatography on a silica gel column ($CHCl_3$/MeOH/TEA, 97.5:0.5:2). Fractions containing **8** were dried by rotory evaporation (*in vacuo*) to give a light yellow foam. Yield 323 mg or 0.29 mmol (89% yield from **7**) TLC ($CHCl_3$/MeOH/TEA, 96:2:2) R_f 0.36. ^{31}P NMR ($CDCl_3$) δ = 153.72, 153.51 p.p.m.

Nucleoside deprotection studies

An aliquot of 250 mg compound **6** was treated with a pyridine/concentrated ammonium hydroxide solution (1:1 v/v) and the deprotection reaction analyzed by monitoring absorbance at 254 nm following analytical reversed phase HPLC on a Rainin C-18 Microsorb column using a gradient of 2–3% CH_3CN (0–12 min), 3–20% CH_3CN (12–20 min), 20–50% CH_3CN (20–30 min) and 50% CH_3CN (30–35 min) in 50 mM sodium phosphate buffer, pH 5.8. Treatment at 55°C for 48 h resulted in **6b** (15%) and **6c** (70%). Both were sensitive to treatment with 20 mM dithiothreitol (DTT) as measured by changes in retention time on HPLC. A minor product (~10%) that was insensitive to DTT treatment was also produced under these conditions, but was not characterized further. Products **6b** (retention time 28.2 min) and **6c** (retention time 26.9 min) were purified by preparative HPLC under conditions identical to those given above except for the omission of sodium phosphate. Further incubation (48 h at 55°C) of **6b** resulted in near complete conversion to the fully deprotected nucleoside **6a**. The structures of **6a**, **6b** and **6c** were confirmed by ^{1}H NMR and mass spectral analysis and each nucleoside showed the expected sensitivity to treatment with the reducing agent DTT. **6a** is 2′-deoxy-6-(cystamine)-2-aminopurine (d^6Cys^2AP); **6b** is

N^2-benzoyl-2′-deoxy-6-(cystamine)-2-aminopurine; **6c** is N^{cys}-benzoyl-2′-deoxy-6-(cystamine)-2-aminopurine.

Oligonucleotide synthesis, purification and characterization

Oligonucleotides were prepared on an Applied Biosystems model 392 DNA synthesizer using standard phosphoramidite chemistry. Commercially available supports (including the Biotin TEG support) and an appropiately protected nucleoside (3′-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidites) were purchased from Glen Research. All other ancillary reagents for automated synthesis of the oligonucleotides were purchased from Prime Synthesis. Phosphoramidite **8** was coupled as a 0.15 M solution using an extended coupling time of 5 min; coupling efficiency was >95%. The 5′-terminal dimethoxytrityl group was removed by the synthesizer and oligomers were cleaved from the support and deprotected by treatment with a solution of concentrated ammonium hydroxide/pyridine (1:1 v/v) at 55°C for 6 h. Oligomers were purified by reversed phase HPLC on a Rainin C-18 Microsorb column using a linear gradient of 2–20% CH_3CN in 50 mM sodium phosphate buffer, pH 5.8. The oligomers were desalted using a C-18 guard column and eluted in 50% aqueous CH_3CN. Oligomers containing **6** were further deprotected at 45°C for 48 h to give oligomers containing **6c**. This purification scheme minimized appearance of the DTT-insensitive species and resulted in good yields. The purity of oligomers was >99% as judged by analytical reversed phase HPLC and/or by PAGE after phosphorylation using polynucleotide kinase and [γ-^{32}P]ATP.

Nucleoside composition of oligomers was determined by digestion of purified oligomers with SVP and SAP (14). The resulting nucleosides were analyzed by reversed phase HPLC using the gradient described in the nucleoside deprotection studies section. Samples of **6a**, **6b** or **6c** from the nucleoside deprotection studies were used as standards.

Sequences

The oligonucleotide substrate was:

5′-CAG ACT GCC G**X**C GCT GCA GGT-3′
3′-GTC TGA CGG CCG CGA CGT CCA-5′

where **X** is either guanine or d^{6}Cys^{2}AP. The substrate used for trypsinization and characterization of peptide bound to DNA was as above except that the d^{6}Cys^{2}AP-containing strand was synthesized using a biotin-containing support. The substrate used to inactivate MTase was:

5′-AGG CT**Y** GAT **Y**CT C**Y**T AC**Y** TGC **Y**TA TAC GAC-biotin-3′
3′-TCC GAC CTA CGA GCA TGC ACG CAT ATG CTG-5′

where **Y** is either guanine or *O*^{6}-methylguanine (O^{6}MeG).

Reduction and labeling of oligomer

Radiolabeling and reduction of oligonucleotides was done simultaneously as follows. An aliquot of 1 pmol oligomer was incubated with 10 µCi [γ-^{32}P]ATP (Amersham), 10 U PNK and 20 mM DTT in a volume of 15 µl for 30 min at 37°C. The kinase was inactivated for 20 min at 65°C with 13 mM EDTA. Unlabeled ATP was then added to 63 nM. A sample of 1.2 pmol complementary strand was added and the strands were annealed by heating to 95°C and slow cooling. Oligomers were separated from unincorporated radiolabel using a G-25 spin column (Boehringer Mannheim) in TE buffer containing 5 mM DTT. The volume was increased to 100 µl to make a 10 nM solution. Oligomers were dialyzed against TE buffer to eliminate any reducing agent that might be left in the solution.

Gel mobility shifts and protein inactivation

Human MTase protein (30 pmol) in TE buffer was added to 1000 c.p.m. labeled substrate (~0.01 pmol) in a volume of 10 µl. The protein/DNA mixture was incubated for at least 5 min before NaCl or DTT was added. Samples were heated to 95°C for 5 min in 2.5% SDS and run on denaturing, non-reducing SDS–PAGE. The resulting gel was dried and exposed to film or to a phosphor screen (Molecular Dynamics) for quantification. To determine whether the active site cysteine was required for binding, the protein was specifically methylated at the active site using an oligomer containing several O^{6}MeG residues. The oligomer was also biotinylated at its 3′-end. Protein (1.4 nmol) was incubated with an equimolar amount of the oligomer (5-fold molar ratio of O^{6}MeG to protein) in a volume of 55 µl for 2 h at 37°C. Buffer was changed to 0.5 M NaCl and 20 mM sodium phosphate, pH 7.4 (avidin binding buffer). UltraLink Immobilized Avidin (Pierce) resin was added to bring avidin to a 2-fold molar excess with respect to biotin and solutions were incubated on ice for 30 min. The resin was spun down and the supernatent dialyzed against TE buffer and used in assays. Mock inactivations were performed with a biotinylated oligonucleotide of the same sequence but containing no O^{6}MeG residues.

Trypsinization of protein–DNA complex

3′-Biotinylated oligomer containing d^{6}Cys^{2}AP was reduced by incubation in 20 mM DTT for 30 min at 37°C and then dialyzed against TE buffer before use. MTase (0.5 nmol) in TE buffer was incubated for 5 min at room temperature with 1 nmol reduced oligonucleotide in a volume of 55 µl. The buffer was adjusted to 10% acetonitrile and 25 mM ammonium bicarbonate, pH 7.4, and the sample incubated at 65°C for 10 min. The sample was then cooled to 37°C, 10 µg trypsin were added and the sample was incubated overnight. Phenylmethylsulfonyl fluoride (0.2 µmol) was added to inactivate trypsin and the sample was left on ice for 15 min. The buffer was adjusted to avidin binding buffer and a 2-fold excess of avidin resin was added. The sample was then incubated on ice for 30 min with periodic agitation. Resin was spun down and washed once with avidin binding buffer and once with 10% acetonitrile. Any peptide still bound was eluted with 0.1 M DTT in 50% acetonitrile and subjected to N-terminal sequencing at the Protein/Peptide Processing Laboratory, Johns Hopkins Medical Institutes. The eluate proved to be a mixture of two peptides. The relative concentration of the two peptides was estimated from amino acid peak heights in the HPLC chromatograms from sequencing cycles 2 and 3. Peak heights were normalized to the heights in a standard solution containing equimolar amounts of the amino acids. Cycle 1 could not be used in this analysis because both peptides start with glycine. Only the next two cycles were used because differences in degradation efficiencies can lead to changes in the apparent ratio.

RESULTS AND DISCUSSION

Phosphoramidite synthesis

Synthesis of the phosphoramidite of d^{6}Cys^{2}AP, **8,** was accomplished with reasonable yield (Fig. 1). Commercially available 2′-deoxyguanosine was protected as the tribenzoylated nucleoside

and the exocyclic oxygen converted to the 2,4,6-triisopropylbenzene sulphonyl derivative as has been described previously (12). Cystamine has been used by others to post-synthetically modify oligonucleotides (8,9) and provided a convenient method for introduction of a disulfide bond into the nucleoside. A solution of **3** was rapidly converted to **4** using an excess of the hydrochloride salt of cystamine in a pyridine/DBU solution. Using an excess of cystamine minimized the possibility for formation of the disubstituted cystamine and excess cystamine was removed by multiple aqueous extraction. The primary amino group of **4** was protected by overnight treatment with benzoic anhydride at room temperature. Use of elevated temperatures in this protection step dramatically decreased the yield of **5** due to the appearance of a new DTT-insensitive species. The 3′- and 5′-*O*-benzoyl protecting groups were removed by mild alkaline hydrolysis, resulting in the protected nucleoside N^2,N^{cys}-dibenzoyl-2′deoxy-6-(cystamine)-2-aminopurine, **6** (Fig. 2). Conversion of **6** to the phosphoramidite **8** was achieved using standard procedures (Fig. 1).

Oligonucleotide deprotection and purification

Several groups have reported that oligonucleotides containing DAP require stringent deprotection conditions (15,16) and, since **6** is an N6 alkyl-modified DAP, we anticipated that special conditions would be required to remove the 2-amino protecting group. In order to optimize deprotection conditions, **6** was deprotected in a solution of ammonium hydroxide/pyridine (1:1 v/v) at 55°C for varying lengths of time. The deprotection reaction was monitored by RP-HPLC. Six hour treatment at 55°C, while sufficient to remove protecting groups from standard commericial nucleosides, resulted in very little conversion of **6** to either **6b** or **6c**. Longer treatments (48 h at 55°C) resulted in the appearance of two new species which corresponded to **6b** (15%) and **6c** (70%). A third species (~10%) also appeared which was insensitive to treatment with DTT; this species could be minimized by deprotecting at 45 instead of 55°C. It was possible to produce the fully deprotected nucleoside **6a**, but this required longer deprotection times at elevated temperatures and was deemed unnecessary, since treatment with DTT of **6c** resulted in the desired thiol-containing nucleoside.

Based on these results, oligonucleotides containing **6** were deprotected and purified in a two step procedure which minimized appearance of the DTT-insensitive species. Protected oligonucleotides containing **6** were deprotected for 6 h at 55°C in an ammonium hydroxide/pyridine (1:1 v/v) solution and the resulting oligonucleotide was purified as described in Materials and Methods. Nucleoside composition analysis showed that oligonucleotide purified at this stage exclusively contained dibenzoylated **6**. Subsequent treatment of **6**-containing oligomers at 45°C for 48 h afforded nearly complete conversion of the oligonucleotide to a species that contained **6c**, the desired product. Figure 3, panel 1 shows the HPLC chromatogram of purified oligonucleotide containing **6c** and change in retention time that occurs as a result of treating this oligonucleotide with 20 mM DTT for 30 min. The nucleoside composition of the oligomer is shown in Figure 3, panel 2 (bottom chromatogram). Note the presence of the peak at 26.9 min, which corresponds to **6c**. Figure 3, panel 2 also shows a duplicate digestion (top chromatogram) that was co-injected with **6c** from the nucleoside deprotection studies in order to show the chromatographic identity of the two.

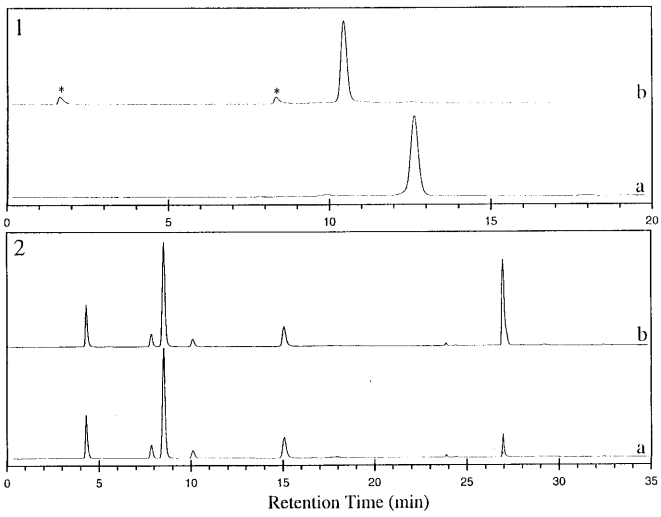


Figure 3. C-18 reverse phase HPLC profiles of purified 21mer containing a single **6c** residue: untreated (panel 1, bottom); reduced with 20 mM DTT (panel 1, top); enzymatically digested to yield free nucleosides (panel 2, bottom); enzymatically digested to yield free nucleosides and co-injected with purified **6c** (panel 2, top). Peaks labeled * in the top chromatogram of panel 1 are present even when only DTT is injected.

Crosslinking between the d^{6}Cys^{2}AP-containing oligonucleotide and human MTase

Human MTase was incubated with radiolabeled oligonucleotide containing the reduced d^{6}Cys^{2}AP-derived nucleoside, denatured in the presence of SDS and high temperature, and run on an SDS–polyacrylamide gel. Control reactions were also performed using an oligomer that contains guanine in place of d^{6}Cys^{2}AP. Figure 4 is an autoradiogram of such a gel. A denaturation-resistant complex is found that is dependent on protein and on the thiol-modified oligomer. The complex does not dissociate in the presence of 0.6 M NaCl, but disappears in the presence of reducing agent (0.2 M DTT). These results suggest that the complex is formed via a disulfide bond.

In order to determine whether the MTase–DNA complex requires the active site cysteine, we specifically methylated this residue by incubating the protein with an oligomer containing O^{6}MeG residues. The inactivated protein did not form a complex with reduced d^{6}Cys^{2}AP substrate, although a control protein, which was mock-inactivated with an unmethylated oligomer, did form a complex (Fig. 4). This requirement for an unmethylated active site cysteine suggests strongly that this cysteine participates in the disulfide linkage. An alternative, if unlikely, possibility is that the active site cysteine does not itself form the disulfide bond with DNA, but is nevertheless required for the crosslink. In this scenario, methylation of the active site cysteine prevents crosslinking by somehow blocking disulfide bond formation between the DNA and other cysteines in the protein. To rule out this possibility, we sought to demonstrate directly that the active site cysteine forms a disulfide bond to the DNA.

Identification of the crosslinked peptide

We synthesized a d^{6}Cys^{2}AP-containing oligonucleotide biotinylated at its 3′-end, reduced the oligonucleotide and allowed it to react

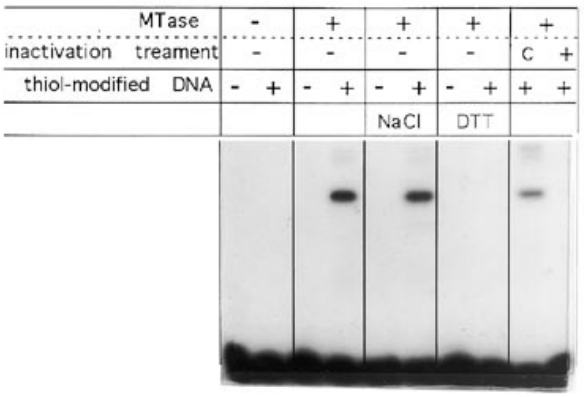


Figure 4. Disulfide bond-mediated crosslinking of human MTase to DNA. Inactivation treatment: +, O^6MeG inactivated; C, mock-inactivated with unmethylated DNA; –, no inactivation treatment. Thiol-modified DNA: +, reduced d^6Cys^2AP-containing oligonucleotide; –, unmodified DNA of the same sequence. NaCl and DTT were added in the lanes indicated as described in Materials and Methods.

with MTase. The complex was then digested overnight with trypsin. Peptide(s) that remained bound to the DNA was purified by binding the biotinylated DNA to avidin-coated beads and collecting the beads by centrifugation. After washing the beads to remove most of the non-covalently bound peptide, the trypsinized peptide that remained associated with the DNA beads was eluted by treatment with 50% acetonitrile and 0.1 M DTT. The first five residues were then determined by Edman degradation. The sequencing reactions confirm that the active site cysteine is directly involved in disulfide bond formation, since the major species identified in the tryptic digest is the peptide that contains the active site cysteine. (Fig. 5 shows the fragments expected for a complete tryptic digest; the active site peptide is underlined.)

A less abundant peptide was also identified in the sequencing reactions. This peptide, which begins with the sequence GTSAA, contains one of the four non-active site cysteine residues and is also, by far, the longest predicted tryptic peptide (Fig. 5). The detection of this other peptide, at ~40% the level of the active site peptide, could indicate the formation of a non-specific disulfide bonded protein–DNA complex. However, we note that no covalent complex was detected at all in the gel mobility assay when the active site cysteine was methylated (Fig. 4). A complex involving a non-active site cysteine could have been detected in this experiment at levels much lower than that suggested by the proteolysis experiment. We think a more likely explanation lies in the more stringent criterion for complex formation used in the gel assay and in the potential for contamination in the proteolysis experiment. First, the gel mobility assay requires that the disulfide bonded complex survive SDS denaturation, whereas the way the proteolysis experiment was done the peptide need only be associated with DNA-coated beads through a relatively mild wash. Thus, an exceptionally large peptide, such as that identified in this experiment, might be detected simply because it is less soluble. Second, the ability of the protein to interact with substrate in a specific manner is lost as the protein is proteolyzed. This means that some of the specificity for an active site disulfide bond that exists at the beginning of the reaction could be lost through disulfide exchange reactions during the extended proteolysis

MDK DCEMK R TTLDSPLGK LELSGCEQGLHEIK LLGK
GTSAADAVEVPAPAAVLGGPEPLMQCTAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTR
QVLWK LLK VVK FGEVISYQQLAALAGNPK ATR AVGGAMR **GNPVP**ILIP**C**HR
VVCSSGAVGNYSGGLAVK EWLLAHEGHR LGK PGLGGSSGLAGAWLK
GAGATSGSPPAGR N

Figure 5. The sequence of human MTase, divided into peptides ending in lysine or arginine. The five amino acids identified by N-terminal sequencing of the peptide that remains crosslinked to DNA following tryptic digestion are shown in bold. The active site cysteine (underlined) is within this peptide.

reaction. It should be noted, however, that only one of the four potential tryptic peptides containing non-active site cysteines is detected.

CONCLUSIONS

We have synthesized oligonucleotides containing d^6Cys^2AP, an analog of guanine, and have shown that a disulfide bond can be formed between the oligonucleotide and the active site cysteine of human MTase. Methylation of this single cysteine appears to abolish the ability to form an SDS-resistant complex despite the existence of four other cysteines in the protein. We interpret this to mean that the active site cysteine is the predominant, if not exclusive, residue with which the oligonucleotide forms a stable disulfide bond. This specificity is especially remarkable given that the active site residue is substantially buried in the crystal structure of *E.coli* Ada (and presumably in the homologous human protein used in these experiments). This inaccessibility implies that there must be a conformational change when the protein binds to and repairs alkylated DNA and it seems likely that the conformational change that allows formation of the disulfide bonded protein–DNA complex will prove similar to that which accompanies DNA repair.

Although the specificity of the reaction suggests that the disulfide bonded complex is similar to the natural complex, there are undoubtedly differences in detail, since the thiol extends two bonds further from the purine ring system than does the methyl or methylene group that is normally transferred to cysteine during the repair reaction. Our choice of the cystamine substituent was based in part on considerations of synthetic simplicity. However, other substituents can also be tried, especially by using 'convertible nucleosides' (11). Convertible nucleosides are nucleoside phosphoramidites with special protecting groups that can be incorporated into an oligonucleotide through normal DNA synthesis procedures. The protecting group can then be replaced with any number of nucleophiles during deprotection of the oligonucleotide. Synthesis of an oligonucleotide with a convertible guanine analog allows different functional groups to be tested without requiring synthesis of a specific phosphoramidite. Indeed, we actually used a convertible nucleoside (Glen Research Inc.) to first test the possibility of crosslinking MTase to DNA through a disulfide bond. Only after obtaining preliminary results with the convertible nucleoside (data not shown) did we turn to the synthesis described here in order to maximize the yield and purity of the desired product.

Only some of the thiol-containing DNA was found to form a covalent complex, even though MTase protein was in excess (Fig. 4). There may be several reasons for this. First, we have not yet made a systematic effort to increase the efficiency of the reaction and there are a number of variations of our protocol that might improve the yield. Second, some of the DNA may be in an

unreactable form due to side reactions or oxidation of the thiol; on the other hand, our characterization of the synthesis products suggest that this is unlikely to be a significant factor. Finally, the length of the linker may impose steric strain on the complex.

In any case, the trapped disulfide-linked complex makes possible certain biochemical studies that would not be possible with the normal reactive complex. If the efficiency of crosslinking can be improved, this analog might also prove useful for structural studies.

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