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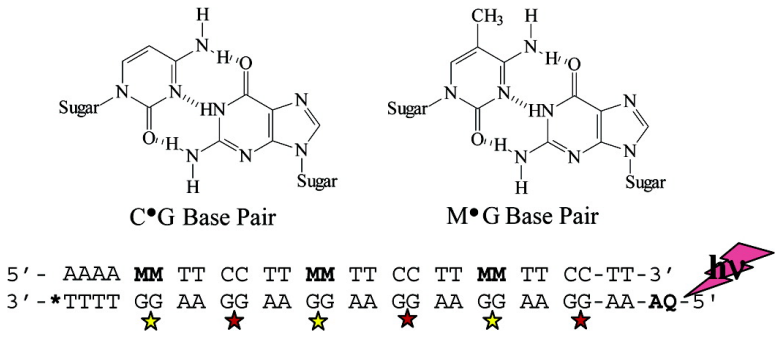

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One-Electron Oxidation of DNA: The Effect of Replacement of Cytosine with 5-Methylcytosine on Long-Distance Radical Cation Transport and Reaction

Sriram Kanvah and Gary B. Schuster*

Contribution from the School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332

Received January 29, 2004; E-mail: gary.schuster@cos.gatech.edu

Abstract: One-electron oxidation of duplex DNA generates a radical cation that migrates through the nucleobases until it is trapped by an irreversible reaction with water or oxygen. The trapping site is often a GG step, because this site has a relatively low ionization potential and this causes the radical cation to pause there momentarily. Modifications to guanine that lower its ionization potential convert it to a better trap for the radical cation. One such modification is the formation of the Watson−Crick base pair with cytosine, which is reported to very significantly decrease its ionization potential. Methylation of cytosine to form 5-methylcytosine (5-MeC) is a naturally occurring reaction in genomic DNA that may be associated with regions of enhanced oxidative damage. The G·5-MeC base pair is reported to be more rapidly oxidized than normal G·C base pairs. We examined the oxidation of DNA oligomers that were substituted in part with 5-MeC. Irradiation of a covalently linked anthraquinone group injects a radical cation into the DNA and results in strand cleavage after piperidine treatment. For the sequences examined, substitution of 5-MeC for C has no measurable effect on the reactions. Cytosine methylation is not a general cause of enhanced oxidative damage in DNA.

Introduction

Cytosine methylation is an enzyme-mediated chemical modification that introduces a methyl group at selected sites on DNA and RNA. In humans and most mammals, DNA methylation occurs primarily at cytosines that are followed by a guanine. Thus, methylated cytosines (5-MeC) occur primarily at clusters of CpG sites in the DNA sequence. Although the function of 5-MeC is not fully understood, it is believed to play a key role in processes such as gene control and cellular differentiation.[1−3] Of particular relevance to this work, Denissenko and co-workers reported that cytosine methylation defines hotspots for oxidative damage to DNA.[4] Oxidation of DNA is suspected of underlying aging and carcinogenesis.[5−7]

One-electron oxidation of DNA introduces a radical cation ("hole") into its stacked nucleobases.[8] In recent years, it has been shown that reactions of radical cations that damage DNA need not occur at the site of initial oxidation. Radical cations in duplex DNA migrate long-distances (hundreds of angstroms) by a reversible hopping process. Trapping of the radical cation occurs by an irreversible reaction with H_2O or O_2 that results in a damaged base.[9]

Because guanine has the lowest ionization potential (I_p) of the nucleobases, it is most commonly damaged in oxidative reactions.[10] In particular, guanines that occur in G_n sequences (where $n = 2, 3$) are particularly prone to reaction because delocalization of the radical cation slightly lowers its energy, which causes it to pause momentarily at these sites.[11,12] In this regard, it has been shown that 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-OxoG) is an especially good trap for radical cations because its I_p is significantly lower than that of guanine.[13] On this basis, we suspected that any structural modification to DNA that lowers the I_p of a guanine should lead to the enhancement of its oxidative reactivity.

The I_p of nucleobases is sensitive to their environment. For example, the I_p of guanosine is lowered by the formation of its Watson−Crick base pair with cytosine. The calculated I_p of the hydrogen-bonded base pair is 0.75 eV below that of guanine itself.[14] Recently, it has been reported that methylation of cytosine reduces its I_p from 8.79 to 8.50 eV,[15] which leads to a rate acceleration for the electrochemical oxidation of its partnered guanine.[16] This suggests that cytosine methylation

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DNA(1)	5′-*ATA TGG TGG TGG T **CGCGC** T GGT GGT GGT-3′
	3′- TAT ACC ACC ACC A **GCGCG** A CCA CCA CCA-**AQ**-5′
DNA(2)	5′-*ATA TGG TGG TGG T **CGMGC** T GGT GGT GGT-3′
	3′- TAT ACC ACC ACC A **GCGCG** A CCA CCA CCA-**AQ**-5′
DNA(3)	5′-*ATA TGG TGG TGG T **MGMGC** T GGT GGT GGT-3′
	3′- TAT ACC ACC ACC A **GCGCG** A CCA CCA CCA-**AQ**-5′
DNA(4)	5′-*ATA TGG TGG TGG T **MGMGM** T GGT GGT GGT-3′
	3′- TAT ACC ACC ACC A **GCGCG** A CCA CCA CCA-**AQ**-5′
DNA(5)	5′-*ATA TGG TGG TGG T **MGMGM** T GGT GGT GGT-3′
	3′- TAT ACC ACC ACC A **GMGMG** A CCA CCA CCA-**AQ**-5′
DNA(6)	5′- AAAA CC TT CC TT CC TT CC TT CC TT CC-TT-3′
	3′-*TTTT GG AA GG AA GG AA GG AA GG AA GG-AA-**AQ**-5′
DNA(7)	5′- AAAA **MM** TT CC TT **MM** TT CC TT **MM** TT CC-TT-3′
	3′-*TTTT GG AA GG AA GG AA GG AA GG AA GG-AA-**AQ**-5′

* = ^{32}P

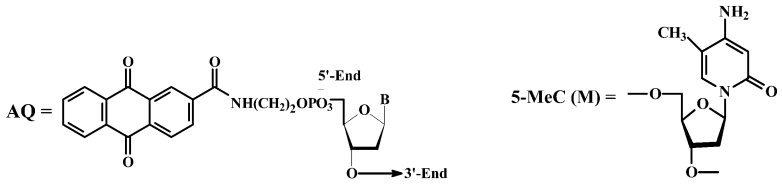

Figure 1. Structures of DNA oligomers.

could affect the efficiency of oxidative damage formation at its paired guanine. The lower I_p of 5-MeC could lead to more efficient trapping of a radical cation at guanine. We tested this hypothesis by examining the efficiency of oxidative damage for a series of DNA oligonucleotides that contain strategically placed 5-MeC nucleobases.

Materials and Methods

TdT enzyme, γ-^{32}P, and α-^{32}P ATP were purchased from Amersham Biosciences. T4 polynucleotide Kinase (T4 PNK) was purchased from New England Biolabs. DNA oligomers and anthraquinone (AQ) containing complementary oligomers were synthesized as described elsewhere[17] on an Expedite 8909 DNA synthesizer. Nucleotide phosphoramidites are obtained from Glen Research and used as received. The extinction coefficients of the oligomers were calculated using a biopolymer calculator, and their concentrations were determined from the absorbance at 260 nm. An adenine is substituted for the anthraquinone group in the extinction coefficient calculation. The oligonucleotides were purified by means of reversed phase HPLC on a Hitachi preparative HPLC system using a Dynamax C18 column. Purified oligomers were desalted and characterized by mass spectroscopy. UV melting and cooling curves were recorded on a Cary 1E spectrophotometer equipped with a multicell block, temperature controller, and sample transport accessory. CD spectra were recorded on a JASCO spectropolarimeter.

Preparation of Radiolabeled DNA. The oligomers were radiolabeled at the 5′-end using γ-^{32}P ATP and T4 PNK enzyme. For radiolabeling at the 3′-end, α-^{32}P ATP and TdT enzyme were used. A 5 μL sample of desired single stranded DNA was incubated with 1 μL of γ-^{32}P [ATP] or α-^{32}P [ATP] and 2 μL of T4 Kinase or TdT enzyme in a total volume of 20 μL at 37 °C for ca. 45 min. After incubation, the DNA sample was suspended in a denaturing loading dye and was purified on a 20% nondenaturing polyacrylamide gel. The desired DNA band was excised from the gel and eluted with 800 μL of elution buffer (0.5 M NH_4OAc, 10 mM $Mg(OAc)_2$/1.0 mM of EDTA/0.1% SDS) at 37 °C for 12 h. The DNA was precipitated from the supernatant by addition of 600 μL of cold ethanol and 2 μL of glycogen. The mixture was vortexed, placed on dry ice for about 60 min, and centrifuged at 12 000*g* for 30 min. The supernatant was removed, and the residual DNA was washed twice with 100 μL of 80% ethanol and air-dried. Suitable volumes of water were added for further experimentation.

UV Irradiation and Cleavage Analysis. Samples for irradiation were prepared by hybridizing a mixture of unlabeled (5.0 μM) and radiolabeled (10000 cpm) oligonucleotides with complimentary AQ-linked DNA in pH 7.0 sodium phosphate buffer solution. Hybidrization was achieved by heating the samples at 90 °C for 10 min, followed by slow cooling to room temperature overnight. Samples were irradiated at ca. 30 °C in microcentrifuge tubes in a Rayonet Photoreactor (Southern New England Ultraviolet Co., Barnsford, CT) equipped with eight 350 nm lamps. After irradiation, the samples were precipitated once with cold ethanol (100 μL) and 2 μL of glycogen. The precipitated samples were washed twice with 100 μL of 80% ethanol, dried, and treated with 100 μL of 1 M piperidine at 90 °C for 30 min. After evaporation of piperidine and coevaporation with water, the samples were dissolved in denaturing loading dye and subjected to 20% 19:1 polyacrylamide gel electrophoresis. The gels were dried, and the cleavage sites were visualized by autoradiography. Quantification of cleavage bands was performed on a Fuji phosphorimager.

Results and Discussion

The DNA oligomers we investigated are shown in Figure 1. Each contains an AQ linked covalently to a 5′-terminus. Irradiation of the AQ "injects" a radical cation into the DNA duplex. In one series we investigated, DNA(1)−DNA(5), each oligonucleotide contains six GG steps that surround a "variable" segment. The variable segment contains five G·C base pairs, and in some cases the cytosines are replaced by 5-MeC. The second oligonucleotides series, DNA(6) and DNA(7), also contain a sequence of six GG steps, but does not have a variable segment. However, some of the cytosines of DNA(6) are replaced by 5-MeC in DNA(7).

The DNA oligomers were purified by HPLC, hybridized in buffer solution, and characterized by their melting behavior (T_m) and by circular dichroism (CD) spectroscopy. The T_m values of DNA(1)−DNA(5) are the same, 66.5 ± 1 °C, and the T_m values of DNA(6) and DNA(7) are 49 and 53 ± 1 °C, respectively. The CD spectra of all DNA samples investigated are similar and characteristic of B-form DNA.[18] Thus, cytosine

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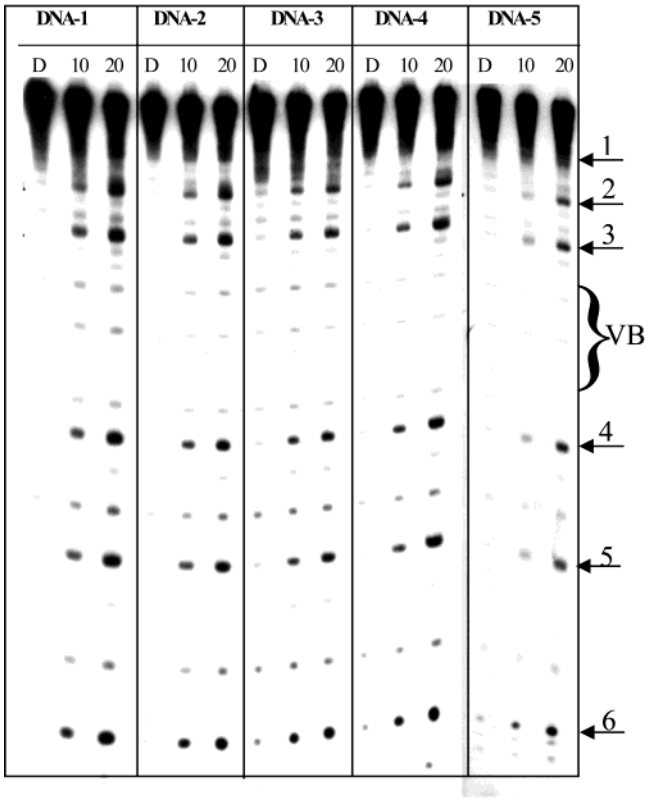

Figure 2. Autoradiogram showing the results of irradiation of DNA(1)−DNA(5). The six GG steps of these DNA oligomers are indicated by the numbered arrows at the right. The three lanes in each gel correspond to 0 min of irradiation (D, dark control), 10, and 20 min of irradiation, respectively. VB identifies the "variable region" of the DNA duplex where 5-MeC are substituted for cytosines.

methylation appears to have only a slight effect on the stability or structure of these DNA oligonucleotides.

It is now well known that one-electron oxidation of the DNA results in reaction at remote GG steps that is revealed as strand cleavage by subsequent treatment of the oxidized DNA with piperidine.[19] A radiolabeled (the * in Figure 1) sample of duplex DNA(1) was irradiated at 350 nm (5 μM, 10 mM sodium phosphate solution, pH = 7.0) and then treated with piperidine for 30 min at 90 °C. Analysis of the irradiation dose dependence shows that "single-hit" conditions are maintained. In this circumstance, each DNA oligomer, statistically, is damaged once or not at all. The irradiated samples of DNA(1) were analyzed by electrophoresis on a denaturing polyacrylamide gel (PAGE) and were visualized by autoradiography. The results are shown in Figure 2.

Strand cleavage is clearly seen at each of the GG steps of DNA(1). As expected, reaction occurs primarily at the 5′-G of each of these GG steps. Moreover, the amount of reaction at GG_2−GG_6 is the same within experimental error (GG_1 is too close to the "heavy spot" for intact DNA to allow meaningful quantification). This result shows that each of the GG steps in DNA(1) react to the same extent and reveals that the rate of radical cation hopping (k_{hop}) is faster than the rate of its irreversible trapping (k_{trap}) by reaction with O_2 or H_2O.[20] Further

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Table 1. Amount of Strand Cleavage Measured by Phosphorimagery at the GG Steps of Oligomers DNA(1)−DNA(7)[a]

GG sequence	DNA(1)	DNA(2)	DNA(3)	DNA(4)	DNA(5)	DNA(6)	DNA(7)
1	ND	ND	ND	ND	ND	≡1.00	≡1.00
2	≡1.00	≡1.00	≡1.00	≡1.00	≡1.00	1.11	1.03
3	1.01	1.02	1.03	1.11	1.12	1.16	0.99
4	1.07	1.07	1.13	1.23	1.06	1.13	1.06
5	1.09	1.09	1.13	1.22	1.05	1.10	1.06
6	1.17	1.19	1.13	1.43	0.97	1.10	1.04

[a] The data represent the ratio of strand cleavage at the identified GG step to GG_1 (GG_2, for DNA(1)−DNA(5)). ND stands for not determined for the reason described in the text.

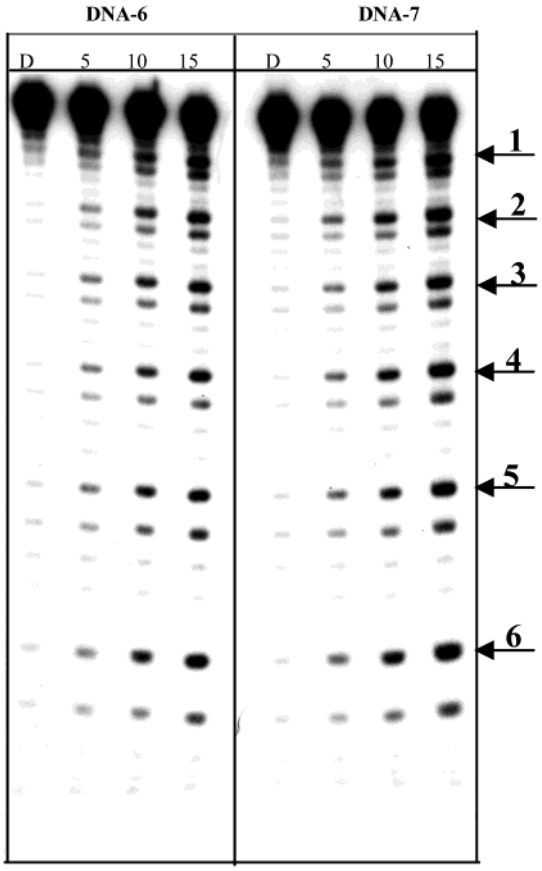

Figure 3. Autoradiogram showing the results of irradiation of DNA(6) and DNA(7). The six GG steps of these DNA oligomers are indicated by the numbered arrows at the right. The four lanes in each gel correspond to 0 min of irradiation (D, dark control), 5, 10, and 15 min of irradiation, respectively.

inspection of Figure 2 shows that there is only a small amount of strand cleavage at the "isolated" guanines (i.e., not part of a GG step) of DNA(1) that are located in the variable region.

In DNA(2), one cytosine in the variable region of the labeled strand is replaced by a 5-MeC. The results of its irradiation are also shown in Figure 2. Within experimental error, the reaction of DNA(2) is identical to that of DNA(1). That is, the 5-MeC substitution does not measurably affect radical cation hopping through the variable region and does not affect the reactivity of adjacent isolated guanines. In DNA(3), there are two 5-MeC groups in the variable region of the labeled strand, and in DNA-(4) there are three. In neither case does 5-MeC substitution affect the hopping of the radical cation or the reactivity of adjacent guanines. In DNA(5), each of the five cytosines in the variable

region is replaced by a 5-MeC, and again there is no measurable difference between DNA(5) and DNA(1). These results also show that 5-MeC substitution does not demonstrably increase the reactivity of isolated guanines paired to them in duplex DNA. The results of quantitative phosphorimagery are shown in Table 1.

DNA(6) has six GG steps that are separated by AA sequences. Its irradiation leads, as expected,[20] to an equivalent amount of strand cleavage (after piperidine treatment) at each site, as shown in Figure 3. In DNA(7), both of the cytosines opposite the guanines in half of the GG steps are replaced by a 5-MeC. The irradiation of DNA(7) shows that there are no measurable differences between the amount of strand cleavage measured between the GG steps that contain cytosines and those having 5-MeC. Also, the reactivities of DNA(6) and DNA(7) are essentially identical. These results show that substitution of 5-MeC for C does not affect the reactivity of GG steps paired to them. The results of quantitative phosphorimagery are shown in Table 1.

The mechanism for long-distance radical cation migration though duplex DNA involves a series of short hops from guanine site to guanine site (where the radical cation briefly pauses) through A·T base pairs. These hops generally occur adiabatically, but may occur by tunneling if the number of bases between the GG steps is less than three.[20−22] In either case, stabilization of the radical cation at a modified G·C base pair will increase the residence time of the radical cation at that site and cause an increase in the amount of strand cleavage. This is observed at GG and GGG sequences where stabilization of the radical cation by delocalization increases the amount of reaction significantly.[11,12] Similarly, substitution of an 8-OxoG for a guanine results in a dramatic localization of the radical cation and decrease in the hopping rate because of the low I_p of this modified base.[17]

There is experimental evidence that the rate constant for electron-transfer quenching by an isolated G·C base pair of triplet *N*,*N*′-dibutylnaphthyldimide in CH_2Cl_2 solution is accelerated from 6.8×10^8 to 8.1×10^8 $M^{-1}s^{-1}$ by changing the cytosine to 5-MeC.[16] This was attributed to transmission of an electronic effect from the 5-MeC to the G through hydrogen bonding of the base pairs.[14] There is a suggestion that these effects might be related to the mutation "hot spots" that are seen in methylated regions of the human P53 gene.[4] However, the results reported here cast doubt on a general connection between this observation and the efficiency of guanine oxidation. The incorporation of 5-MeC in the DNA oligomers we examined does not measurably affect either the migration of radical cations through those modified base pairs or the trapping of guanines at these modified sites. This means that the effect of 5-MeC substitution on k_{hop} is so small that it does not significantly affect the ratio k_{hop}/k_{trap}. This ratio of rate constant depends on the specific sequence of base pairs separating GG steps.[20] Consequently, there may be special circumstances where substitution of 5-MeC changes reactivity, but this is not a general phenomenon.

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