Effect of C5-Methylation of Cytosine on the Photoreactivity of DNA: A Joint Experimental and Computational Study of TCG Trinucleotides

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ⓈSupporting Information

ABSTRACT: DNA methylation, occurring at the 5 position of cytosine, is a natural process associated with mutational hotspots in skin tumors. By combining experimental techniques (optical spectroscopy, HPLC coupled to mass spectrometry) with theoretical methods (molecular dynamics, DFT/TD-DFT calculations in solution), we study trinucleotides with key sequences (TCG/T5mCG) in the UV-induced DNA damage. We show how the extra methyl, affecting the conformational equilibria and, hence, the electronic excited states, increases the quantum yield for the formation of cyclobutane dimers while reducing that of (6-4) adducts.

Methylation, occurring at the 5 position of cytosine (C), is a natural process that plays an important role in epigenetic regulation of gene expression.[1] Although 5-methylcytosine (5mC) represents <5% of the bases in the human genome,[2] it is associated with 30% of the mutational hotspots in skin tumors.[3,4] The increased mutation rate, in particular C→T transitions observed at T5mCG sites, was correlated with the formation of T5mC cyclobutane dimers (CPDs, Scheme 1), which after deamination gives rise to thymine CPDs in larger yield than with non-methylated cytosine.[5,6] This overall effect, being particularly important for

UVB irradiation, was attributed to the 6 nm red-shift of the cytosine absorption spectrum upon C5-methylation.[7] But this spectral change cannot account for the CPD yields observed upon UVC irradiation of isolated genomic DNA in solution.[8] To get insight into the enhanced photoreactivity of T5mCG sequences compared to their non-methylated counterparts, we have undertaken a thorough investigation of the photoactivated processes in TCG and T5mCG trinucleotides based on photoproduct analysis by HPLC coupled to mass spectrometry, optical spectroscopy, molecular dynamics (MD) simulations, and DFT/TD-DFT calculations in solution. Here, we show that conformational changes in the ground state observed upon C5-methylation can explain the quantum yields of dimeric photoproducts (Scheme 1), CPDs formed in the $\pi\pi^*$ state, and 64PP, whose formation involves a $T^+\rightarrow C^-$ charge-transfer state. Moreover, we show that C5-methylation similarly affects the cytosine conformational behavior in the isolated trimer and in the double strand, where other factors, for example energy transfer,[9] render a full description of the photoinduced processes much more delicate.

First, we examined whether C5-methylation of our model systems has the same effect on UV-induced reactions as in the case of genomic DNA. The quantitative effect of C5-methylation on the photoproduct yield is still a matter of debate.[8,10,11] To address this issue, in contrast to all the previous studies, instead of "yields" we determined "quantum yields" (ϕ, Table 1). Following irradiation at 254 nm, we found indeed higher ϕ_{CPD} values for T5mCG, whereas the opposite

Scheme 1. Formation of T5mC Dimeric *c,s* and *t,s* Photoproducts[a]
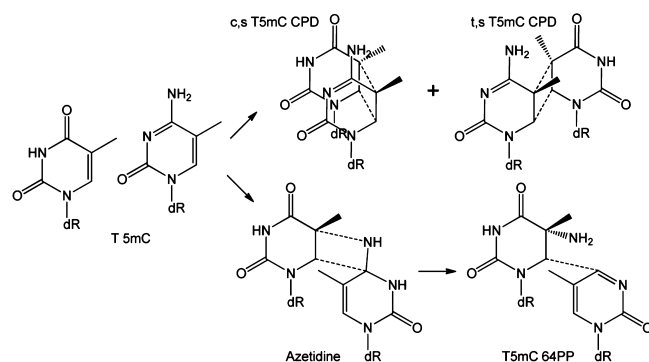
[a]CPD, *cis,syn* and *trans,syn* cyclobutane pyrimidine dimers; 64PP, pyrimidine (6-4) pyrimidone photoproducts; dR, 2-deoxyribose.

Table 1. Quantum Yields ϕ of Formation of Dimeric Photoproducts Determined Following Irradiation at 254 nm

	ϕ_{TCG}[a]	ϕ_{T5mCG}[a]	I_{C5}[b]
$\phi_{CPD(c,s)}$ (×10³)	0.50 ± 0.09	0.75 ± 0.09	1.72
$\phi_{CPD(t,s)}$[c] (×10³)	0.46 ± 0.07	0.87 ± 0.11	2.17
ϕ_{64PP} (×10³)	0.63 ± 0.04	0.44 ± 0.01	0.80
ϕ_{CPD}/ϕ_{64PP}	1.52	3.68	

[a]Commercially available, HPLC purified, dissolved in phosphate buffer. [b]$I_{C5} = \phi_{T5mCG}\psi_{TCG}/\phi_{TCG}\psi_{T5mCG}$, where ψ represents the fraction of photons absorbed by the cytosine moiety in each system. [c]Summing also deaminated photoproducts.

was observed for ϕ_{64PP}. The intrinsic effect of C5-methylation is better revealed after normalization of ϕ, considering the fraction of photons absorbed at 254 nm by the cytosine moiety in each system (0.23 for TCG, 0.20 for T5mCG; see SI). According to this analysis, the increase in $\phi_{CPD(c,s)}$ upon methylation, not due to the shift of the absorption spectrum, amounts to ∼70%. Note that such analysis requires knowledge of the molar absorption coefficients of all the chromophores; this is possible for trinucleotides, but so far it cannot be achieved for isolated genomic DNA and, even less, for cellular DNA. The context-dependent photon absorption associated with energy-transfer processes precludes quantitative comparison between results obtained for these systems.[8,10,11]

Next, we discuss the experimental absorption and fluorescence spectra of the trimers in the light of results obtained by MD and quantum mechanical (QM) calculations. The temperature dependence of the absorption spectra of TCG and T5mCG (Figure 1a), corrected for thermal changes
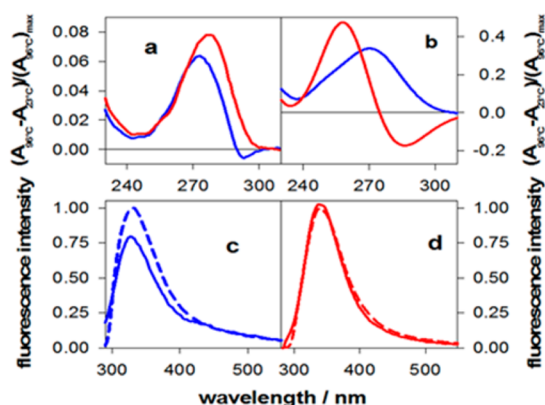

Figure 1. Comparison of the spectral properties of TCG (blue) and T5mCG (red). (a) Differential absorption spectra obtained by subtracting the spectrum at 23 °C from that at 96 °C, and (b) the corresponding spectra calculated for c2c1c1 conformers, uniformly shifted for easier comparison with experiments (see SI). (c,d) Fluorescence spectra obtained following excitation at 255 nm; dashes correspond to non-interacting mononucleotides; relative intensities are proportional to the fluorescence quantum yields.

observed for the monomeric chromophores[12,13] (Figure S2), although weak, reveals the existence of stacked conformers, whose population decreases upon increasing temperature.

MD simulations (two independent 250 ns long runs, for each trimer), performed using a computational protocol successfully applied to the study of oligonucleotides,[14] show indeed that, at room temperature, at least two bases per trimer are stacked during 50−70% of the simulation time; structures with all three bases stacked are also frequently encountered, especially in the case of TCG. Subsequently, two representative conformers of TCG and T5mCG were studied by PCM/M052X calculations, which have already been successfully used in the study of TT photoreactivity.[15,16] We found that destacking of all three bases is reflected in the absorption spectra (Figures 1b, S7, and S8), in line with the experimental spectra.

C5-Methylation affects the fluorescence spectra of the trimers (Figure 1c,d). Although the spectrum of TCG, peaking at 330 nm, indicates emission from $\pi\pi^*$ states of the monomeric chromophores,[17] the fluorescence quantum yield is ∼20% lower than that of the stoichiometric mixture of mononucleotides. In contrast, the T5mCG spectrum coincides,

within the experimental error, with that of its monomeric constituents both in shape and in intensity. According to computations at the PMC/TD-M052X level, the observed difference in the fluorescence behavior of the two trimers can be explained on the basis of their different propensity to evolve toward $G^+{\rightarrow}C^-$ charge-transfer states during the excited-state relaxation; population of such dark states induces a decrease in the quantum yield of the $\pi\pi^*$ fluorescence. Our results strongly suggest that this channel is more effective for CG compared to 5mCG, in agreement with the partial quenching of $\pi\pi^*$ fluorescence in the non-methylated trimer (Figure 1c). PCM/M052X calculations on dinucleotides indicate indeed that C5-methylation increases significantly the stability of the TC stacking, with a much smaller effect on CG stacking, leading to a relative destabilization of CG stacking. MD simulations of the trimer structure provide a similar picture: the CG step is stacked during 75% of the simulation time, but for the 5mCG step stacking corresponds only to 52%. As C5-methylation decreases the percentage of CG stacked structures, it reduces the mixing between the bright excited states of the monomers with the $G^+{\rightarrow}C^-$ charge-transfer state. In longer sequences, especially when in double strands, C5-methylation is expected to have a smaller effect on the percentage of CG stacked structures. Deactivation to a $G^+{\rightarrow}C^-$ CT state is favored for non-methylated dinucleotides, independent of their conformation (see SI). This is due both to a larger energy gap between the 5mC and G excited states and to a slightly smaller electron affinity of 5mC compared to that of C.

Our QM calculations show that several effects contribute to the larger photochemical reactivity of 5mC. First, as discussed above, C5-methylation increases TC stacking. Furthermore, the conformational preferences of the stacked structures are significantly affected by the presence of the extra methyl. As shown in Table 2 (see also SI), in the most stable conformer of

Table 2. Relative Energy (kcal/mol) and Selected Geometrical Parameters (Å) of the Main Conformers of TC and T5mC, Determined by PCM/M052X/6-31+G(d,p)// PCM/M052X/6-31G(d) Calculations

	conformer			
	c3c3	c2c2	c2c1	c2c4
		TC		
ΔE	0.0	−0.07	0.17	−0.09
d	3.99	4.56	4.34	3.98
g	3.62	3.95	3.55	3.66
		T5mC		
ΔE	0.0	0.95	0.58	0.78
d	3.90	4.57	4.10	4.11
g	3.69	4.00	3.35	3.44

d, distance between the midpoints of C5C6 bonds; g, distance between the C5 of thymine and the nitrogen of the amino group of C/5mC.

T5mC, the two sugars adopt a C3′endo−C3′endo puckering (hereafter c3c3). In contrast, in the case of TC, conformers in which thymidine adopts C2′endo puckering dominate, with C2′endo−C4′exo (c2c4) being the most stable conformer. Furthermore, the C2′endo−C1′exo (c2c1) conformer is more stable than the C2′endo−C2′endo (c2c2) one for T5mC, while the opposite is found for TC. Thus, C5-methylation induces a decrease of the pseudo-rotation phase angle, measuring the ring puckering, of the sugar.

A picture similar to that found by QM calculations regarding conformational preferences emerges also from MD simulations, despite the fact that the latter method strongly underestimates the stability of c3c3 conformations (see SI). Indeed, TC adopts c2c2 and c2c1 puckering during 32% and 25% of the simulation time, respectively. For T5mC the relative frequency is reversed: ~40% for c2c1 and 25% for c2c2. As in c2c2 conformers, the C5 substituent of C is too close to the C2′ group of the preceding base (Figure 2); the presence of a bulky methyl
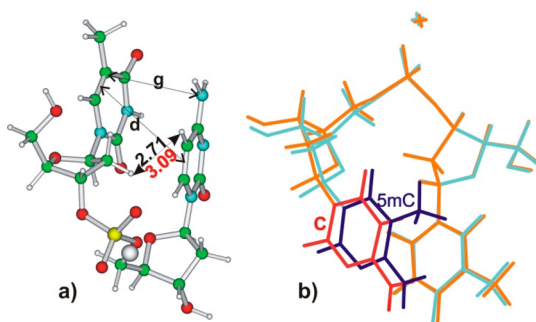

Figure 2. (a) Schematic drawing of TC-c2c2, derived from QM calculations, highlighting the C5−C′2 contact. The distance between a H atom on C′2 and that on C5 for TC-c2c2 (black) and TC-c2c1 (red) is reported. (b) Superposition between TC-c2c1 (orange/red) and T5mC-c2c1 (cyan/blue).

group in this position leads to the onset of nonbonded repulsion. The latter is relieved by a decrease in the phase (c2c2 → c2c1), inducing an increase in the distance as found for T5mC steps. Even when considering the same conformer, C5-methylation leads to small but noticeable structural changes. For c2c1 conformers, small backbone rearrangements lead to a shift of the 5mC base, allowing better stacking with T (Figure 2), mirrored by a decrease of the distance d between the midpoints of C5−C6 bonds. MD simulations provide the same trend: the average d value determined for T5mCG is shorter than that found for TCG. We stress that it has been shown that small d values are strongly correlated with the reactivity toward CPD formation,[18,19] though in our studies of TpT we have shown that this factor is less important than sugar puckering.[15,16]

The above-described structural features significantly affect the photochemical behavior of the examined species. In the case of TC-c3c3 and T5mC-c3c3 conformers, which are characterized by a short d in the S_0 minimum, PCM/TD-M052X calculations predict that a barrierless path on the potential energy surface (PES) of the lowest energy exciton state leads to a pseudo-minimum. This point, characterized by very short C5−C5 and C6−C6 distances, is an intermediate in the reactive path leading to CPD (Figure 3a). CPD is then formed, without any significant energy barrier, through an effective S_1/S_0 conical intersection, analogous to that discussed for TT.[20−23] Similar results are obtained for c2c1 conformers (see SI), whereas for c2c2 conformers our calculations predict localization of the excitation on the C/5mC nucleotide and a "monomer-like" decay. These findings explain why ϕ_{CPD} is higher for methylated trimers (Table 1), which exhibit a preference for c3c3 and c2c1 conformers.

From the above we conclude that the sugar puckering, which modulates the "global" stacking arrangement of the bases, governs the formation of TC CPDs, thus explaining the influence of C5-methylation. This is in line with what is
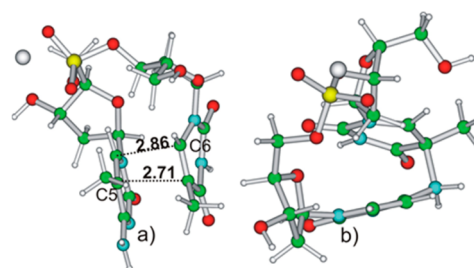

Figure 3. (a) Pseudo-minimum in the PES of the lowest energy excited state of Tp5mC-c3c3, reporting C5−C5 and C6−C6 distances. (b) Representative structure of the crossing region between the PES of the T$^+$→C$^−$ charge-transfer state with S_0, leading to the azetidine-like intermediate.

reported for TT photodimerization, showing that sugar puckering plays a key role in the quantum yield of the dimeric photoproducts. For TpT analogues, where the sugars are locked in C3′endo conformation by a methylene bridge, ϕ_{CPD} is much larger, and 64PP production is inhibited.[24] In this respect, it is noteworthy that the relative stability of the c3c3 conformer, for which CPD formation is favored,[24,25] is larger for TT (>1.7 kcal/mol)[16] than for T5mC (<1 kcal/mol), in line with the higher CPD yield in TT dinucleosides found experimentally.[26] Finally, in view of the above-described reaction path leading to CPDs, involving $\pi\pi^*$ states, the lengthening of the fluorescence lifetime observed upon C5-methylation of the nucleoside[12] may also contribute to the enhanced reactivity of the methylated system.

We also performed the first QM calculations describing the path leading to TC 64PP. We located on the PES of the T$^+$→C$^−$ charge-transfer state a crossing region with S_0, where a bond between C5 and the nitrogen (N4) of the amino group of cytosine is almost formed. These findings indicate that, in analogy with what was found for TT,[15,16] a charge-transfer state involving the transfer of an electron from the 5′ to the 3′ base is critical for the formation of an azetidine-like species (Figure 3b), the counterpart of the oxetane intermediate in the path leading to TT 64PP. The puckering corresponding to the crossing region is c2c4, which is more stable for TC than for T5mC, in line with the decrease in ϕ_{64PP} observed upon C5-methylation (Table 1). Interestingly, the distributions of the distance g (Figure 2) derived from MD trajectories show that the percentage of conformers where g is <4.0 Å is higher for TCG than for T5mCG. This distance is the counterpart of the O4−C5 distance in TT steps, which has been proposed as diagnostic for 64PP formation.[19]

Our MD simulations indicate that the sugar−phosphate backbones of trimers, although sampling a wide range of conformations, mainly correspond to B-DNA structures. In addition, we performed a thorough MD analysis of the duplex 5′-CG(TCGTA)₃CG-3′ and its hemi-methylated analogue (see SI). We found that the conformation of TCG/T5mCG steps is not significantly altered by its inclusion in the double strand. More importantly, C5-methylation induces a decrease of the average sugar phase angle, leading to a shift of the C puckerings from c2 toward c1 and o4, in line with our MD results on the single-stranded trimer as well as the results of previous studies.[27]

In the present study, we examined the effect of C5-methylation on the conformational equilibria in trinucleotides, tackling key sequences for the UV-induced DNA damage

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(TCG/T5mCG). We showed that the presence of a methyl group at the 5 position of C induces structural changes which can be directly related to their photochemical behavior: methylation strengthens interaction with flanking T, disfavors stacking with an adjacent G, and stabilizes conformers that are more reactive toward CPD formation. Moreover, for the first time, the effect of C5-methylation on the conformational and photochemical properties of TC was explored by first-principleS QM calculations including the phosphoribose backbone. We demonstrated that C5-methylation increases the quantum yield of CPD formation, which occurs on the PES of the lowest energy exciton state, and modulates the decay to a charge-transfer state with the flanking G. Moreover, it decreases the quantum yield of 64PP formation, taking place on the PES of a $T^+{\rightarrow}C^-$ charge-transfer state. The results of our calculations are in agreement with both the experimental absorption and fluorescence spectra and the quantum yields of dimeric photoproducts determined for the examined trinucleotides. Finally, our MD study of duplexes containing TCG/T5mCG sequences indicates that these structural factors should be also operative in double-stranded DNA.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Additional experimental and computational details and results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

■ ACKNOWLEDGMENTS

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