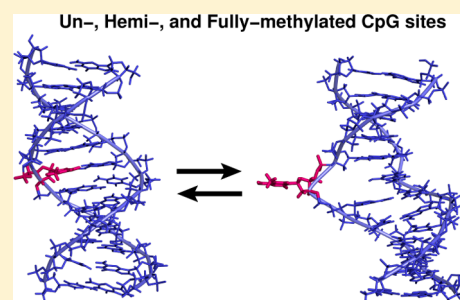


Base-Flipping Propensities of Unmethylated, Hemimethylated, and Fully Methylated CpG Sites

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S Supporting Information

ABSTRACT: Methylation of C5 of cytosines at CpG dinucleotide sites of the DNA is one of the most important factors regulating the expression of genes. The interactions of these CpG sites with proteins are essential for recognition and catalysis and in many cases are characterized by the flipping of either of the cytosine bases out of the DNA helix. In this paper, we present results from molecular dynamics simulations indicating that methylation of CpG sites suppresses spontaneous extra-helical conformations of either of the two cytosines. Thus, cytosines in unmethylated sites flip out easier than in hemimethylated sites and the latter flip out easier than in fully methylated sites. The different propensities for base flipping is observed not only between the cytosines that differ in their methylation states but also between the cytosines on the complementary strand. From alchemical mutation calculations, we find that methylation of one of the cytosines increases the free energy of the extra-helical conformation by 10.3–16.5 kJ/mol and this increase is additive with respect to the second methylation. Potential of mean force calculations confirm these results and reveal that cytosines in unmethylated sites favor flipping via the major-groove pathway. We perform several analyses to correlate this behavior with structural changes induced by the different methylation states of the CpG site. However, we demonstrate that the driving force for these propensities is the change in the electronic distribution around the pyrimidine ring upon methylation. In particular, unmethylated cytosine interacts more favorably (primarily via electrostatic forces) with solvent water molecules than methylated cytosine. This is observed for, both, extra-helical cytosines and intra-helical cytosines in which the cytosine on the complementary strand flips out and water molecules enter the DNA double-helix and substitute the hydrogen bonds with the orphan guanine. On the basis of these results of spontaneous base flipping, we conjecture that the mechanism for base flipping observed in complexes between hemimethylated DNAs and proteins is not likely to be passive.



I. INTRODUCTION

In the DNA double helix structure, the bases on one strand are paired with the complementary bases on the other strand. This conformation is quite stable, primarily due to the hydrogen bonds between the paired bases and the π -interactions of the stacking bases along the DNA helix. Although the former interactions can be, to some extent, compensated by hydrogen bonds with the solvent water molecules, the π -stacking interactions are completely lost if a base changes its intra-helical (flipped-in) conformation to an extra-helical (flipped-out) state. Therefore, an energetic penalty is involved with flipped-out bases. Nevertheless, vital biological processes depend on the ability of certain nucleotides to flip out. These processes require extra-helical conformations mainly because it is easier for an enzyme to catalyze reactions on a flipped-out base. Examples are found in the bound structures of a DNA with cytosine-5¹ and adenine^{2,3} methyltransferases, thymine-dimer^{4,5} and 8-oxoguanine⁶ repair enzymes, uracil-DNA glycosylase,⁷ and endonuclease IV.⁸ In addition, flipped-out bases can participate in RNA splicing and ribozyme reactions.^{9,10} However, base flipping is not involved only in

catalysis but also in the recognition of hemimethylated CpG sites by UHRF1^{11–14} and in signaling the termination of mitochondrial transcription.¹⁵

In all of these protein–DNA bound complexes, the flipped-out base is inserted into the binding pocket of the protein. How does this sequence of events proceed? Does the flipping out of the nucleotide precede the binding to the protein, or vice versa? Schemes for both of these pathways have been proposed. In the “passive” mechanism, the base spontaneously flips out of the DNA helix and only then the protein recognizes the extra-helical nucleotide and binds to it. In contrast, in the “active” pathway, the protein binds to the DNA first in a conformation in which the base is intra-helical, and this binding facilitates the flipping out of the recognized nucleotide.

Experimental evidence of both mechanisms is reported in the literature. The X-ray structure obtained for a methyltransferase–DNA bound complex in which the cytosine to be methylated

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exhibits an intermediate flipped-out conformation¹⁶ supports the notion that the enzyme actively participates in the base-flipping process. In contrast, recent studies have shown that the uracil repair-enzyme slides along short stretches of DNA, searching for uracil base, while the latter is in an extra-helical position.^{17,18} Nevertheless, the reports may not need to contradict each other. Spontaneous base flipping of a DNA is significantly more probable for mismatched or damaged bases than for the usual Watson–Crick, G:C and A:T, base pairs that are characterized by optimal hydrogen bondings and π -stacking.^{19–24} Likewise, nucleotides in RNA bulges were shown to exhibit a larger propensity for base flipping than bases that are paired.^{20,25–27} Thus, it is possible that the mechanism of base flipping is not universal; depending on the tendency of the nucleotide to flip out, the pathway can be either active or passive.

Studies based on NMR imino proton exchange have shown that spontaneous (nonterminal) base-pair opening occurs in unbound DNA in solution.^{28–33} It is a thermally activated process, as evidenced by the decrease in the flipping rate for lower temperatures. In general, the lifetime of the flipped-in state correlates with the strength of interaction between the base pair; within the canonical B-DNA structure, the lifetime of a G:C base pair (10–50 ms) is found to be about 10 times longer than that for an A:T base pair (1–5 ms). The lifetime of the flipped-out state is on the order of nanoseconds; thus, the equilibrium constant between the extra-helical and intra-helical states is on the order of 10^{-6} – 10^{-7} . More information obtained from these experiments is that the base-flipping event of a nucleotide is not strongly correlated to that of its neighbors, and only moderately dependent on the sequence of the DNA.³⁴ There are, however, some exceptions. The most pronounced are the 1 order of magnitude retardation in the opening rate of thymine within A-tract sequences,^{30,35} and the acceleration in the opening rate of guanine by tracts of G:C base pairs.³⁶ Sequence dependent base-flipping rates were also observed in other systems lacking these two sequence patterns.³⁷ Note that imino proton exchange experiments are argued to measure only partial opening because the base needs only to open enough for the imino protons to exchange with the acceptor base from the solvent.³⁸

When observed, the sequence dependent base-flipping rates are attributed to structural changes the B-DNA structure experience. For example, in A-tracts, there is a narrowing of the minor groove that also influences the flexibility of the major groove.³⁹ This raises the question, toward which groove of the DNA is it energetically more favorable to flip out a base? Computer simulation studies were dominating in addressing this question.³⁸ The picture that emerged from the majority of the studies is that, for the smaller pyridine bases, cytosine and thymine, flipping through the major and minor grooves is energetically comparable. However, for the larger purine bases, guanine and adenine, a preference for opening through the major-groove pathway is observed.^{20,25,40–47} This is because flipping via the minor-groove direction is more difficult due to steric hindrance of the backbone atoms. Flipping via the major-groove direction is also inferred from experimental results.⁴⁸ When a DNA (in the intra-helical conformation) is bound to a protein, the base flipping is reported to be more favorable in the direction of the major groove even for the smaller pyrimidine bases.^{24,49}

From a thermodynamic point of view, chemical modifications of nucleotide bases can either stabilize or destabilize the

flipped-out conformation. It has been recently reported that derivatives of adenine and cytosine tethering a phenyl or naphthyl group exhibit a larger propensity for extra-helical conformations relative to the unmodified bases.⁵⁰ Central to epigenetics is the chemical modification of cytosine bases. In the DNA of mammals, this epigenetic mark is a methyl group covalently attached to cytosine (at position C5) in the dinucleotide sequence CpG. Base flipping of the (target) cytosine to be methylated has been observed^{1,51} by X-ray crystallography for the catalysis of the methylation reaction.^{52–54} In addition, base flipping of the cytosine on the complementary strand has also been observed.^{11–14} In this case, the extra-helical state is necessary for the recognition of hemimethylated CpG sites by the protein UHRF1 (hemimethylated sites emerge after the replication of methylated sites and are characterized by the methylation of only one of the two cytosines). UHRF1 then signals the enzyme Dnmt1 to catalyze the methylation reaction on the target cytosine. Does methylated cytosine exhibit a different tendency for base flipping than unmethylated cytosine? Experimental studies indicate that methylation of N6 of adenine, in the GATC site, changes the equilibrium between the extra-helical and intra-helical conformations of the two A–T base pairs.⁵⁵ The A–T base-pair dissociation constants in the unmethylated GATC site are, approximately, twice as large as those in hemimethylated GATC site, and the latter are, approximately, twice as large as those in the fully methylated GATC site. Thus, methylation of adenine reduces the propensity for base flipping and the effect on the equilibrium constant is additive. No effect has been observed on the base opening of the neighboring, unmodified, G:C and C:G base pairs. In addition, on the basis of experiments using NMR imino proton exchange, it is argued that the retardation in the base-opening rate of thymine within A-tract sequences arises due to the C5 methyl group of thymine.⁵⁶

In this paper, we investigate the propensity for spontaneous base flipping of cytosines in different methylation states of CpG dinucleotide sites. We find that C5 methylation of cytosine destabilizes the extra-helical conformation of either of the cytosine bases of this site and the effect is additive with respect to a second methylation. It is shown that this behavior is a result of a change in the electronic distribution around the cytosine ring due to the methylation reaction.⁵⁷ An unmethylated cytosine interacts more favorably with the surrounding solvent water molecules than methyl-cytosine and, therefore, can compensate more for the penalty of its extra-helical position or that of the cytosine on the complementary strand.

II. METHODS

The DNA sequence simulated in this work is a dodecamer that was used experimentally to investigate the interactions of unmethylated, hemimethylated, and fully methylated CpG sites with the regulatory protein UHRF1.¹¹ The recognized CpG site is located halfway along the chain at positions C6pG7 and the corresponding bases on the complementary strand are C7'pG6' (see Figure 1). The initial structure of this dodecamer taken for the simulations was an ideal B-DNA double helix conformation built using the PREDICTOR software.⁵⁸

The DNA and counterions were represented by the AMBER99 force-field⁵⁹ and the solvent water molecules by the TIP3P model.⁶⁰ The partial charges of 5-methylcytosine, which are not available in the standard parameters of the

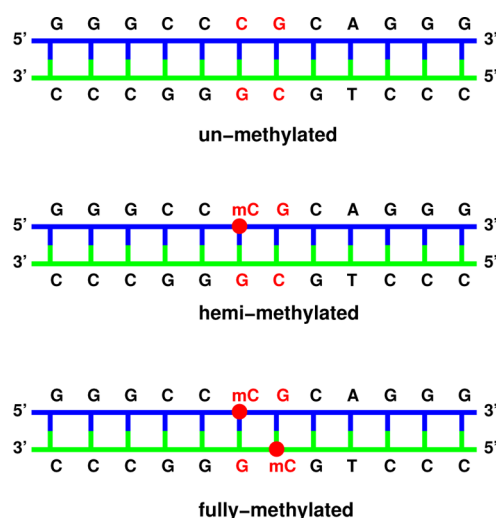


Figure 1. The sequence of the DNA double helix that was simulated in this study. The CpG site (labeled in red) is located in the middle of the DNA chain at positions 6 and 7 (6' and 7' on the complementary strand). The base mC denotes cytosine base that is methylated at position 5 of its pyrimidine ring.

AMBER99 force-field, were taken from the work of Rauch et al.⁶¹ These charges were obtained from an ab initio calculation using the restrained electrostatic potential (RESP) charge fitting procedure.⁶² For comparison we also performed simulations in which the DNA was modeled by the parmbsc0 force-field.⁶³ The shape of the simulation box was cubic, and its size was determined by imposing a minimum distance of 1.2 nm between the DNA atoms and each of the box walls. This box was then solvated by 8356 water molecules. Because the 12-mer DNA contains 22 negatively charged phosphate groups, we neutralized the system by adding 22 sodium cations at random positions.

The molecular dynamics package GROMACS⁶⁴ version 4.0.7 (4.5.5 for the parmbsc0 force-field) was used to perform all of the computer simulations with a time step of 0.002 ps and periodic boundary conditions applied in all three dimensions. The simulations employed the velocity rescaling thermostat,⁶⁵

with a coupling time of 0.1 ps, to maintain a constant temperature of 300 K. In addition, the Berendsen barostat⁶⁶ with a compressibility of 5×10^{-5} 1/bar and a coupling time of 1.0 ps was also employed to maintain the system at a constant isotropic pressure of 1 bar. Long-range electrostatics interactions were calculated using the particle mesh Ewald method^{67,68} with a real-space cutoff of 1.0 nm, grid spacing of 0.12 nm, and quadratic interpolation. The Lennard-Jones potential was calculated using a 1.0 nm cutoff. The covalent bond distances of the DNA were constrained using the LINCS algorithm,⁶⁹ whereas water bond distances and angles were constrained using the SETTLE algorithm.⁷⁰ The system was first energy minimized using the steepest descent algorithm, followed by a 2 ns simulation in which the positions of the DNA heavy atoms were restrained by a harmonic potential with a force constant of 1000 kJ/(mol·nm²). Then, 10 ns of unrestrained simulation was performed. The configurations that emerged from these simulations for the unmethylated, hemimethylated, and fully methylated systems were used as an input for the free energy calculations for the flipped-in state.

We define the flipped-out state as a conformation of the DNA double helix in which either C6 or C7' is extra-helical. To generate the conformation of the flipped-out bases, simulations with a slow-growth technique were performed. In order to flip the C6 base via the major and minor grooves, a pseudodihedral angle, θ , defined by the atoms C4(C6)–P(C6)–P(G7)–C2(G7), was changed in 10^5 steps (i.e., in 200 ps) from 0 to $\pm 180^\circ$ using a restrained potential of 4000 kJ/(mol·nm²). In these slow-growth simulations, the system configuration was saved every 2 ps. A value of $\pm 180^\circ$ of the opening angle corresponds to a flipped-out conformation in which C6 points away from the double helix⁴⁹ (see Figure 2). In an ideal B-DNA conformation, the value of this pseudodihedral is $\theta = 10.33^\circ$. Flipping the base on the complementary strand (C7') involved changing the corresponding pseudodihedral angle, C4(C7')–P(C7')–P(G6')–C2(G6'), that in an ideal B-DNA conformation equals $\theta = 4.18^\circ$. To ensure that the base-flipping process does not disrupt the hydrogen bonds between the base pairs above (C5:G5') and below (G7:C7') the flipped-out base, we applied in this preparatory stage position restraints, with a force

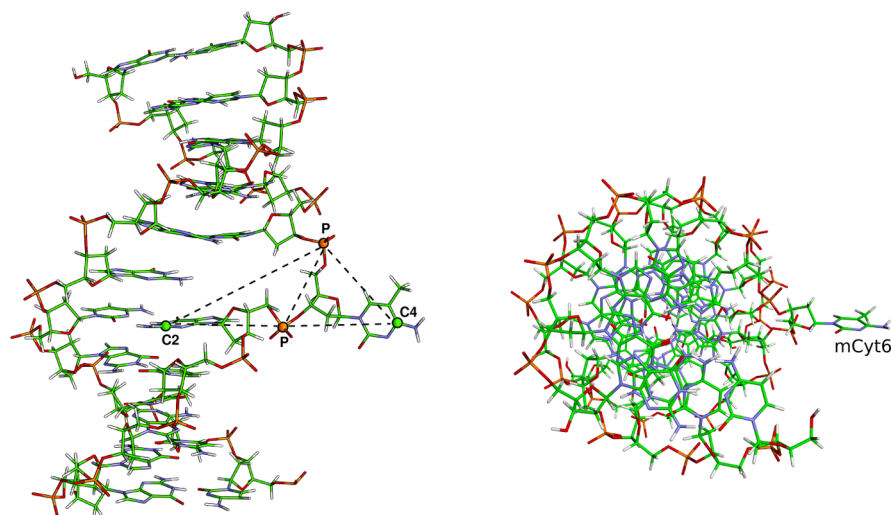


Figure 2. A snapshot from the alchemical mutation simulations of side and top views of a conformation of the flipped-out state of C6 in hemimethylated DNA. The atoms defining the pseudodihedral are labeled, and the two planes they form are shown by dashed lines. In this particular conformation, the value of the pseudodihedral is 180.7° .

constant of 1000 kJ/(mol·nm²), on the heavy atoms that are associated with these Watson–Crick hydrogen bonds. In all subsequent simulations, these position restraints were removed. Nevertheless, in the flipped-out state, the restraints on the atoms defining the pseudodihedral at $\theta = \pm 180^\circ$ were kept with a force constant of 3000 kJ/(mol·nm²). The systems were then equilibrated for 2 ns, and the last frames obtained were used as the starting configurations for the mutation free energy transformations of the flipped-out states.

Mutation Free Energy Calculations. The relative free energy changes for the base-flipping processes were computed by the concept of a thermodynamic cycle⁷¹ (see Figure 3). To

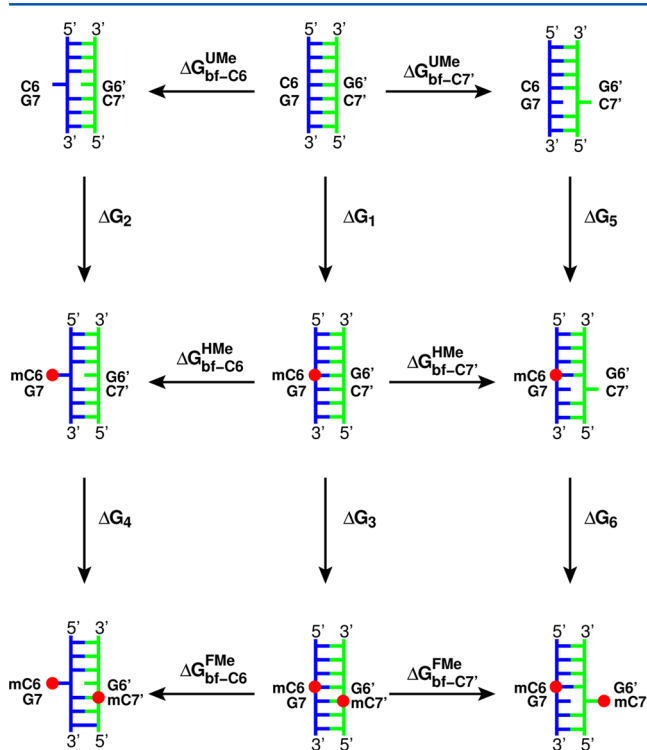


Figure 3. The thermodynamic cycles constructed to calculate the relative free energy changes of flipping out the (m)C6 base (left arrows) and (m)C7' base (right arrows) for unmethylated (top), hemimethylated (middle), and fully methylated (bottom) CpG sites.

this end, alchemical mutations of atom types (with soft-core potentials, $\alpha = 0.7$ and $p = 1$), bonds, angles, and dihedrals were performed to transform unmethylated cytosine to methylcytosine and vice versa.⁷² More specifically, in the forward direction, H5 of cytosine and three dummy atoms covalently bonded to it were mutated to a carbon atom and three hydrogen atoms, thus transforming cytosine to methylcytosine, whereas in the backward transformation C5 and the three methyl hydrogens were mutated to a hydrogen atom and three dummy atoms, respectively. These transformations were performed for both the flipped-in and flipped-out states. The free energy changes associated with these transformations were computed by the thermodynamic integration technique.⁷³ For each transformation, 11 equally spaced λ -points from $\lambda = 0$ to $\lambda = 1$ were constructed. At each λ -point, the system was equilibrated for 5 ns and then data collected for another 25 ns. For the mutations in which the plot of $\partial H/\partial \lambda$ as a function λ did not exhibit a smooth curve, we added up to four λ -points. For all transformations, we calculated the free energy change in the

forward and backward directions. All structural analyses for the unmethylated, hemimethylated, and fully methylated states were averaged over the forward and backward transformations, i.e., for 50 ns.

Potential of Mean Force Calculations. To characterize the free energy profile along a reaction coordinate that represents a base-flipping process, we calculated the potential of mean force (PMF) of flipping (m)C6 in unmethylated and hemimethylated CpG sites. The reaction coordinate for constructing the PMF is defined by the pseudodihedral, θ , described above for the slow-growth simulations (see Figure 2). This base-opening angle was changed from 0 to $\pm 180^\circ$ through 21 θ -points in each direction. Negative and positive values of θ represent opening via the major and minor grooves of the DNA, respectively. For each θ -point, the starting configuration was taken from the slow-growth simulations, equilibrated for 10 ns, and then data collected for an additional 30 ns. Different base-opening angles correspond to different values of the coupling parameter λ , and the reported values are averages at each λ -point over the data-collection segment of the trajectory. The average force needed to restrain θ to a particular value (using a force constant of 3000 kJ/(mol·nm²)), i.e., $\langle \partial H/\partial \lambda \rangle$, is then integrated as a function of λ to obtain the PMF. As the PMF represents only relative values, it was shifted such that the free energy of the equilibrium flipped-in state corresponds to zero.

The estimation of the errors of the free energy changes was obtained by integrating the error estimated at each λ -point. The error at each λ is calculated by dividing the standard deviation of $\partial H/\partial \lambda$ by the square root of the number of independent data points. The latter was estimated from the total time of the simulation for each λ divided by the autocorrelation time of $\partial H/\partial \lambda$.⁷⁴ Because we calculated the electrostatic interactions using Ewald summation, the decomposition of the potential energy into contributions from different groups was performed by the direct evaluation of the interparticle distances from the trajectories (using the *-rerun* option in Gromacs).

III. RESULTS AND DISCUSSION

Alchemical Mutation Free Energy Calculations. We constructed four thermodynamic cycles to investigate the relative free energy change of flipping out each of the two cytosines in unmethylated, hemimethylated, and fully methylated CpG sites. These cycles are shown in Figure 3. The free energy change of the individual alchemical transformations (i.e., the transformation of a hydrogen atom to a methyl group, and vice versa) as well as the relative free energy changes for the base-flipping processes are given in Table 1. These results indicate that the propensity of an unmethylated cytosine to flip out of the DNA helix is larger than that of a methyl-cytosine. This is true for flipping out (m)C6, $\Delta \Delta G_{bf-C6}^{UMe-HMe} = -14.3$ kJ/mol, and for flipping out (m)C7', $\Delta \Delta G_{bf-C7'}^{HMe-FMe} = -10.3$ kJ/mol. In addition, flipping out a (methyl)-cytosine base is easier when the second (flipped-in) cytosine is unmethylated than if it is methylated. This can be seen in flipping out mC6, $\Delta \Delta G_{bf-C6}^{HMe-FMe} = -16.5$ kJ/mol (-12.8 kJ/mol when using ΔG_4^*), and in flipping out C7', $\Delta \Delta G_{bf-C7'}^{UMe-HMe} = -12.5$ kJ/mol. Given the magnitude of the error estimates, all of these relative free energy changes are within the range of one another and it is not clear whether the variation observed is significant. Thus, methylation at position 5 of cytosine (in a CpG site) destabilizes either its extra-helical position or the other cytosine's extra-helical position, and the effect is observed to

Table 1. The Free Energy Changes of the Alchemical Mutations Shown in Figure 3^a

	forward	backward	average
ΔG_1	$+435.9 \pm 4.4$	$+436.4 \pm 5.2$	$+436.1 \pm 3.4$
ΔG_2	$+451.0 \pm 4.0$	$+449.8 \pm 3.0$	$+450.4 \pm 2.5$
ΔG_3	$+447.8 \pm 5.1$	$+448.4 \pm 5.3$	$+448.1 \pm 3.7$
ΔG_4	$+464.7 \pm 4.8$	$+464.6 \pm 4.8$	$+464.6 \pm 3.4$
ΔG_4^*	$+462.9 \pm 4.7$	$+458.9 \pm 4.7$	$+460.9 \pm 3.3$
ΔG_5	$+450.8 \pm 4.8$	$+446.4 \pm 4.6$	$+448.6 \pm 3.3$
ΔG_6	$+455.3 \pm 3.3$	$+461.5 \pm 3.0$	$+458.4 \pm 2.2$
$\Delta\Delta G_{bf-C6}^{UMe-HMe} = \Delta G_1 - \Delta G_2$			-14.3 ± 5.9
$\Delta\Delta G_{bf-C6}^{HMe-FMe} = \Delta G_3 - \Delta G_4$			-16.5 ± 7.1
$\Delta\Delta G_{bf-C6}^{HMe-FMe} = \Delta G_3 - \Delta G_4^*$			-12.8 ± 7.0
$\Delta\Delta G_{bf-C7'}^{UMe-HMe} = \Delta G_1 - \Delta G_5$			-12.5 ± 6.7
$\Delta\Delta G_{bf-C7'}^{HMe-FMe} = \Delta G_3 - \Delta G_6$			-10.3 ± 5.9

^aWe also calculated ΔG_4 with the parmbsc0, amber refined, force-field, ΔG_4^* . The relative free energy changes for flipping either of the two cytosines (C6 or C7') in the different methylation states of the CpG site are also indicated. All values are given in kJ/mol.

be additive. This behavior is qualitatively the same as that reported experimentally for flipping either of the two adenine bases in different methylation states (of adenine) of GATC sites.⁵⁵

Potentials of Mean Force. Independent of these alchemical mutation simulations, we calculated the PMF of flipping (m)C6 in unmethylated and hemimethylated CpG sites, thus, the processes corresponding to ΔG_{bf-C6}^{UMe} and ΔG_{bf-C6}^{HMe} in Figure 3, respectively. Figure 4 displays the PMFs for

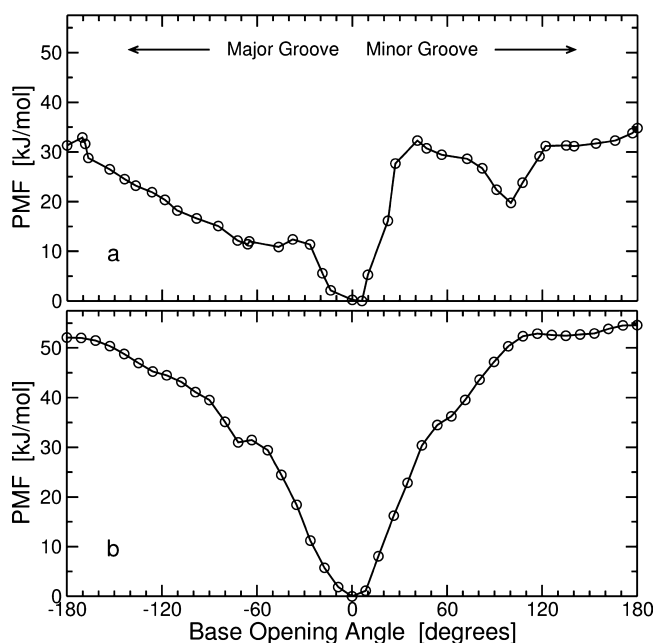


Figure 4. The potential of mean force of flipping out (a) the cytosine C6 in the unmethylated CpG site (corresponding to the process of ΔG_{bf-C6}^{UMe} in Figure 3), and (b) the methylcytosine mC6 in the hemimethylated CpG site (ΔG_{bf-C6}^{HMe} in Figure 3). Positive values of the base-opening angles correspond to flipping via the minor groove, whereas negative values correspond to flipping via the major groove.

opening the flipped-in conformation ($|\theta| \approx 0^\circ$) to the completely flipped-out conformation ($|\theta| \approx 180^\circ$) via, both, the major and minor grooves. Six snapshots of these base-flipping processes via the major-groove pathway are shown in Figure 5 for unmethylated and hemimethylated CpG sites. The free energy changes accompanying these base flippings are given in Table 2. For each methylation state, these changes in free energy are independent of the path and, therefore, should be equal. The results shown in Table 2 indicate that these values are within 3.5 kJ/mol from each other and that the error in determining each of the free energy changes is larger. On average, the flipped-out states are destabilized by 32.9 and 53.4 kJ/mol for the unmethylated and hemimethylated CpG sites, respectively, relative to the flipped-in state. Thus, $\Delta\Delta G_{bf-C6}^{UMe-HMe} = -20.5 \pm 6.7$ kJ/mol, which is within the range of the -14.3 ± 5.9 kJ/mol obtained by the mutation free energy calculations.

In addition to the different propensities of unmethylated and hemimethylated CpG sites to adopt an extra-helical conformation, the shape of the two PMFs is also different. The curve for hemimethylated is more or less symmetrical with respect to the flipped-in state, and no preference for base-opening via either pathway is observed. However, for the unmethylated CpG site, major-groove opening is more probable than the route via the minor groove. For both curves, the hydrogen bonds within the C6:G6' pair are maintained for $|\theta| \lesssim 45^\circ$ and the deviations from the equilibrium value are represented by a harmonic (quadratic) shape.⁴⁵ However, in the unmethylated case along the major-groove opening, the PMF exhibits a small "shoulder" at around $\theta = -45^\circ$ with a free energy level that is significantly lower than that of the minor groove with a similar degree of base-opening angle. This free energy height is also much lower than that in the corresponding points in the hemimethylated curve and is a result of hydrogen bonds the (partially) flipped-out cytosine base (C6) makes with two adjacent nucleotides C5 and C7' (in addition to the hydrogen bonds it partially forms with its paired base G6'). We calculated that C6 in unmethylated DNA, at these base-opening angles, makes 1.1 more intra-DNA hydrogen bonds than in hemimethylated DNA. In the latter, only partial compensation by hydrogen bonds (of about 0.5) with the solvent is observed. Once the flipping base, C6, in the major-groove pathway of the unmethylated DNA loses most of its pairing to G6' (at around $\theta = -65^\circ$), it assumes a parallel (to the helix axis) orientation stabilized by the formation of a hydrogen bond between its NH_2 group and the oxygen of the phosphate group of the adjacent nucleotide C5 (we display this behavior in Figure 6). At larger values of the base-opening angle (around $\theta = -70^\circ$), we also observed a hydrogen bond between N4 of C6 and the hydrogen of the same group (NH_2) of the other adjacent nucleotide, C7' (also here partial pairing of one hydrogen bond between C6 and G6' was still observed). In contrast, the flipping cytosine in the hemimethylated DNA at the corresponding base-opening angles forms a much smaller number of hydrogen bonds with the adjacent nucleotides. Furthermore, in the range $-165^\circ \lesssim \theta \lesssim -120^\circ$ (major groove), we observe that in the unmethylated case the base can also adopt occasionally a conformation in which it is parallel to the axis of the helix (see Figure 5 at $\theta \approx -140^\circ$). This conformation is also a result of the intra-DNA hydrogen bond C6 makes with the oxygen of the phosphate group of C5 and is in equilibrium with the perpendicular orientation that is observed exclusively at larger $|\theta|$. Similar behavior occurs also in the minor-groove pathway at around $\theta = 100^\circ$ which explains

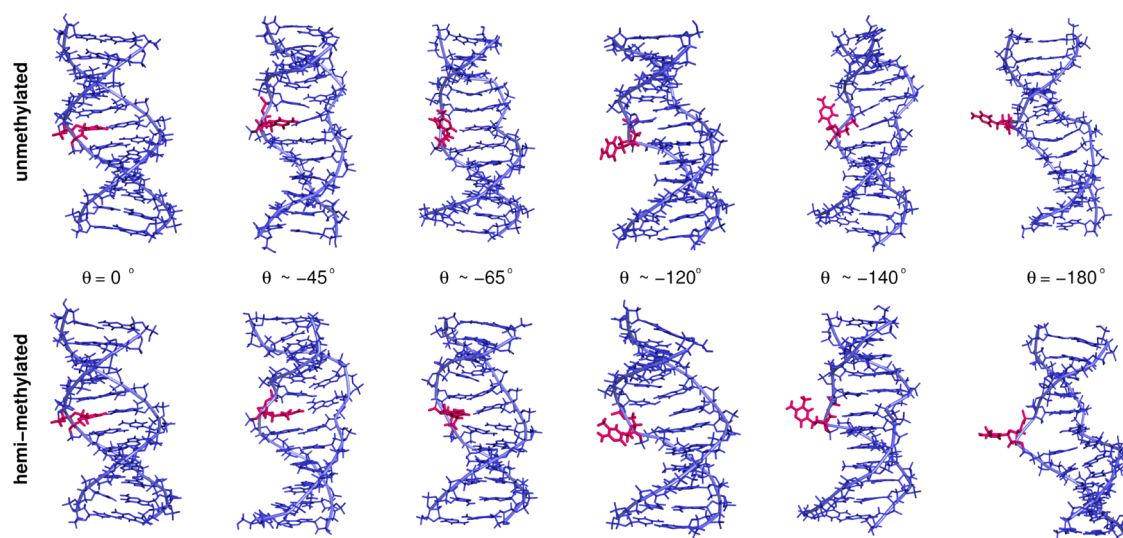


Figure 5. Snapshots from the PMF trajectories displaying the orientation of the flipping base (m)C6 (colored in red) with respect to the B-DNA double helix. These instantaneous configurations exhibit the gradual flipping of (m)C through the major-groove pathway, thus for decreasing values of the base-opening angle θ , for the unmethylated and hemimethylated CpG sites.

Table 2. Results Extracted from the PMF Curves Shown in Figure 4 of the Free Energy Change for (m)C6 to Adopt an Extra-Helical Conformation ($|\theta| \approx 180^\circ$) from Its Equilibrium Intra-Helical Conformation ($|\theta| \approx 0^\circ$)^a

	major groove	minor groove	average
ΔG_{bf-C6}^{UMe}	$+31.1 \pm 4.7$	$+34.6 \pm 4.8$	$+32.9 \pm 3.4$
ΔG_{bf-C6}^{HMe}	$+52.1 \pm 4.3$	$+54.6 \pm 5.1$	$+53.4 \pm 3.3$
$\Delta\Delta G_{bf-C6}^{UMe-HMe}$			-20.5 ± 6.7

^aThis change in free energy is calculated for flipping via both the major- and minor-groove pathways in unmethylated and hemimethylated CpG sites. For comparison with the alchemical mutation free energy calculations (Table 1), the value of $\Delta\Delta G_{bf-C6}^{UMe-HMe}$ is also computed. All values are given in kJ/mol.

the local minimum observed at that location. For the hemimethylated CpG site, this parallel orientation is hardly

observed; nonpaired intra-DNA hydrogen bonds were detected only at $\theta = -80^\circ$. The small “shoulder” observed in this case at around $\theta = -70^\circ$ signifies the onset of hydrogen bonds of C6 and G6' with the solvent water molecules.

Why Does Methylation Suppress Extra-Helical Conformations? Given the observations described above, we conjecture that the preference for flipping unmethylated cytosine via the major-groove pathway is not due to steric constraints *per se* but is a result of larger possibilities for intra-DNA hydrogen bonds which stabilize partially opened states. In the Supporting Information, we describe and present results (Table S1) demonstrating that the widths of the major and minor grooves are also not correlated with the free energy propensities of flipping out the cytosine bases. Furthermore, the change in the populations of the BI and BII states upon

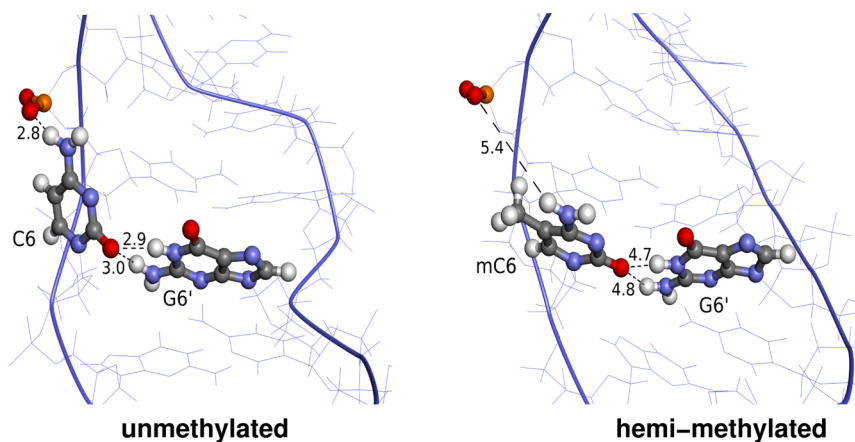


Figure 6. The hydrogen bonds the partially flipped-out cytosine base ($\theta \approx -65^\circ$) makes with its base-pair guanine as well as with the oxygen atom of the phosphate group of C5 in the unmethylated CpG site. The latter hydrogen bond (with a probability of 0.70) is the reason the partially flipped-out cytosine adopts (more or less) a parallel to the helix orientation. For hemimethylated DNA, the formation of this non-base-paired intra-DNA hydrogen bond is much lower (0.26) and is likely to be the reason for the more perpendicular orientation of the partially flipped-out base. Note that in these trajectories the number of base-paired hydrogen bonds is 1.7 and 1.0 for unmethylated and hemimethylated CpG sites, respectively. The numbers indicated correspond to distances (in Å), averaged over the entire data-collection segment of trajectories, between a donor oxygen and a hydrogen acceptor.

Table 3. The Average Number of Hydrogen Bonds the Four Bases of the CpG Site Make with Either the Solvent Water Molecules or the DNA (Both Strands)^a

	(m)C6 extra-helical			(m)C7' extra-helical		
	unmethylated	hemimethylated	fully methylated	unmethylated	hemimethylated	fully methylated
(m)C6–water	4.7 ± 0.2	4.5 ± 0.2	4.5 ± 0.4	1.8 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
G6'–water	5.0 ± 0.7	5.5 ± 0.2	5.0 ± 0.6	3.9 ± 0.3	3.9 ± 0.2	4.0 ± 0.2
(m)C7'–water	1.8 ± 0.2	1.8 ± 0.1	2.4 ± 0.8	4.7 ± 0.2	5.0 ± 0.1	4.3 ± 0.1
G7–water	3.7 ± 0.4	3.9 ± 0.1	3.9 ± 0.2	5.0 ± 0.4	5.5 ± 0.1	5.7 ± 0.2
(m)C6–DNA	0.2 ± 0.2	<0.1	<0.1	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1
G6'–DNA	0.8 ± 0.4	<0.1	0.8 ± 0.8	3.1 ± 0.1	3.2 ± 0.1	3.1 ± 0.1
(m)C7'–DNA	3.1 ± 0.1	3.1 ± 0.1	1.7 ± 0.6	0.1 ± 0.2	<0.1	<0.1
G7–DNA	3.1 ± 0.1	3.1 ± 0.1	2.5 ± 0.4	0.6 ± 0.4	<0.1	<0.1

^aThe calculations are performed on the, alchemical mutations, trajectories in which the conformation of (m)C6 is extra-helical, as well as for those in which the conformation of (m)C7' is extra-helical. A hydrogen bond is defined by a donor–acceptor cutoff distance of 0.35 nm and a donor–hydrogen–acceptor angle larger than 150°.

methylation⁷⁵ cannot explain the relative free energy changes for base flipping either (see Table S2, Supporting Information).

Another possible interpretation for the flip-out propensities obtained is that the 5-methyl group enhances the base-pair G:C interaction in the flipped-in state. We calculated the number of hydrogen bonds between the cytosine to be flipped out and the rest of the DNA in the flipped-in conformation. However, the values obtained for unmethylated, hemimethylated, and fully methylated CpG sites are essentially the same, in the range 3.1–3.2, reflecting the stability of the two G:C base pairs. Additionally, methylation can disrupt the interaction of the cytosine that flips out, or of the orphan guanine, with the water molecules. In Table 3, we calculated the number of hydrogen bonds each of the four bases in the CpG site makes with the surrounding waters. For the two flipped-out states, the results indicate that also in this case there are no significant differences between the number of hydrogen bonds the bases, in the different methylation states of the CpG site, make with the solvent water molecules. We also display in Table 3 the number of intra-DNA hydrogen bonds associated with the CpG sites in the extra-helical states. Here, there is a small difference in the number of hydrogen bonds involving the orphan guanine (thus, G6' when (m)C6 is extra-helical and G7 when (m)C7' is extra-helical); however, it is on the margin of the error and is not likely to be significant. However, a noticeable reduction in the G7:C7' base-pair hydrogen bonds is observed in the fully methylated CpG site. We analyzed the conformation of this fully methylated DNA and found a deformation of the helix coupled to a loss of the base-pair hydrogen bonds. A snapshot of this deformation is shown in Figure S1 in the Supporting Information. This deformed conformation was able to recover at later times to the undeformed B-DNA with a complete base-pair hydrogen bond in one out of the two alchemical mutation trajectories. The alternations between these two (deformed and undeformed) conformations were also observed in some of the other λ -points in the vicinity of the fully methylated state. To address the role of the force-field on this behavior, we also performed two other simulations of this fully methylated system with the parmbsc0 force-field⁶³ for 30 ns. In this case, the G7:C7' base-pairing persisted throughout these two trajectories (see Figure S1, Supporting Information). Due to the different behavior observed for the two force-fields, we calculated ΔG_4 shown in Figure 3 also with the parmbsc0 force-field. However, as indicated in Table 1, no significant difference in the value of the free-energy change is observed, confirming

the conclusion that methylations of a CpG site suppress the extra-helical conformations of the cytosines.

What is then the physical mechanism responsible for the reduced base-flipping propensities with increasing the degree of methylation of a CpG site? In mutating cytosine to methylcytosine (or vice versa), there are two main changes. The first is the increase of the excluded volume, representing the growth of a methyl group. The second is the change in the charge distribution around the pyrimidine ring due to the methylation reaction. To address the contribution of each of these changes to the difference in the free energy observed in Table 1, we calculated $\Delta\Delta G_{bf-C6}^{UMe-HMe}$ by a two-step transformation via an intermediate (cavity) state (see Figure 7). In this intermediate state, the partial charges of the cytosine (to be mutated to methylcytosine) are the same as those of unmethylated cytosine; however, instead of H5, we constructed an atom (cavity) that has the same excluded volume as a methyl group (the charge and Lennard-Jones dispersion interactions of this cavity atom were kept as those of H5; see Figure S2 (Supporting Information) in our previous work⁵⁷). The results are shown in Table 4.

Via this intermediate state, $\Delta\Delta G_{bf-C6}^{UMe-HMe} = -13.3$ kJ/mol, very similar to the value found via the direct transformation (-14.3 kJ/mol). The analysis of the two subcycles indicates that the creation of the excluded volume of the methyl group is negligible; $\Delta G_{bf-C6}^{UMe} - \Delta G_{bf-C6}^{cavity} = -0.6$ kJ/mol. However, almost the entire magnitude of $\Delta\Delta G_{bf-C6}^{UMe-HMe}$ arises predominantly from the change of the partial charges of the pyrimidine ring due to the methylation at C5; $\Delta G_{bf-C6}^{cavity} - \Delta G_{bf-C6}^{HMe} = -12.7$ kJ/mol.

Upon methylation, there is a weakening of the interaction energy between the cytosine base and the surrounding water molecules. This is because of a change in the distribution of the electrons (which is manifested in the change of the partial charges) around the pyrimidine ring of cytosine accompanying the methylation reaction.⁵⁷ Obviously, in the extra-helical conformation, the cytosine that is flipped out interacts with the surrounding waters more than when it is base-paired in its intra-helical state. Therefore, this weakened interaction upon methylation is more pronounced for extra-helical conformations. To demonstrate this behavior, we calculate the potential energy between the cytosine that is mutated in the processes shown in Figure 3 and the rest of the system (thus, DNA, waters, and ions). The results are shown in Table 5. Although the magnitudes of the estimated errors are large, it is possible to draw few conclusions from these results. The values of ΔE_1 and

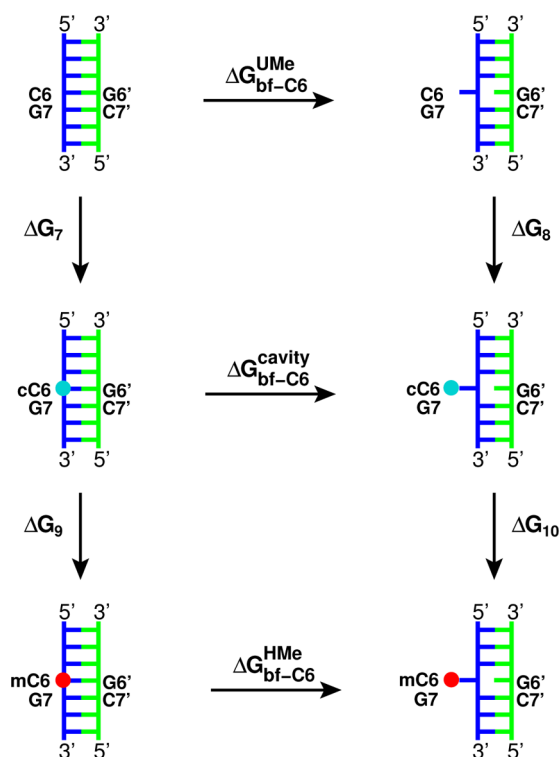


Figure 7. The thermodynamic cycle showing the calculation of the free energy change of flipping out mC6 in hemimethylated DNA relative to flipping out C6 in unmethylated DNA via an intermediate cavity state. In this intermediate state, the partial charges of the flipped-out cytosine are the same as those of unmethylated cytosine; however, instead of H5, we constructed an atom (cavity) that has the same excluded volume as a methyl group (see text).

Table 4. The Free Energy Changes Associated with the Transformations via the Intermediate-Cavity State Shown in Figure 7^a

	forward	backward	average
ΔG_7	$+15.9 \pm 0.2$	$+15.9 \pm 0.3$	$+15.9 \pm 0.2$
ΔG_8	$+16.6 \pm 2.2$	$+16.4 \pm 2.0$	$+16.5 \pm 1.5$
ΔG_9	$+439.5 \pm 4.0$	$+438.8 \pm 4.6$	$+439.1 \pm 3.0$
ΔG_{10}	$+453.6 \pm 2.7$	$+450.0 \pm 3.2$	$+451.8 \pm 2.1$
$\Delta \Delta G_{bf-C6}^{UMe-HMe} = \Delta G_7 - \Delta G_8 + \Delta G_9 - \Delta G_{10}$			-13.3 ± 6.8

^aThe free energy change for flipping the (m)C6 base in unmethylated DNA relative to that in hemimethylated DNA is also indicated. The values of ΔG_7 and ΔG_9 were taken from our previous study,⁵⁷ and the same procedures were applied for the calculations of ΔG_8 and ΔG_{10} . All values are given in kJ/mol.

ΔE_3 are significantly lower than the corresponding values of the other processes. In these two processes, both cytosines are in their intra-helical conformation, confirming the smaller extent of the cytosine–solvent interactions. For the other processes, in which one of the cytosines is extra-helical, the changes in the energies are all positive, demonstrating that methyl-cytosine interacts with water less favorably than cytosine. The stronger interaction of unmethylated cytosine with water is not only when this cytosine is extra-helical (ΔE_2 and ΔE_6). It is also evident when the mutated cytosine is intra-helical and the other cytosine in the CpG site is extra-helical (ΔE_4 and ΔE_5). In this case, water molecules do enter the double-helix structure of the DNA to form hydrogen bonds with the orphan guanine and

Table 5. The Changes in the Potential Energy between the Cytosine That Is Mutated and the Rest of the System for the Transformations Shown in Figure 3^a

	τ_R (ns)	τ_P (ns)	average
ΔE_1	140	140	-0.1 ± 11.6
ΔE_2	140	140	$+13.4 \pm 11.8$
ΔE_3	200	60	$+3.7 \pm 21.6$
ΔE_4	60	60	$+16.7 \pm 19.9$
ΔE_4^*	60	60	$+14.6 \pm 22.5$
ΔE_5	60	120	$+12.3 \pm 5.9$
ΔE_6	120	60	$+30.9 \pm 21.9$
$\Delta \Delta E_{bf-C6}^{UMe-HMe} = \Delta E_1 - \Delta E_2$			-13.5 ± 23.4
$\Delta \Delta E_{bf-C6}^{HMe-FMe} = \Delta E_3 - \Delta E_4$			-13.0 ± 41.5
$\Delta \Delta E_{bf-C6}^{HMe-FMe} = \Delta E_3 - \Delta E_4^*$			-10.9 ± 44.1
$\Delta \Delta E_{bf-C7'}^{UMe-HMe} = \Delta E_1 - \Delta E_5$			-12.4 ± 17.5
$\Delta \Delta E_{bf-C7'}^{HMe-FMe} = \Delta E_3 - \Delta E_6$			-27.2 ± 43.5

^aAnalogous to the results shown in Table 1, we also calculated ΔE_4 with the parmbsc0 force-field, ΔE_4^* . The averages for each state were calculated over all the trajectories, thus, for the alchemical mutations (in the forward and backward directions) as well as from the potential of mean force calculations. The values τ_R and τ_P indicate the total simulation times taken for calculating the average of each process for the reactant and product sides, respectively. The relative potential energy changes for flipping either of the two cytosines (C6 or C7') in the different methylation states of the CpG site are also indicated. All values are given in kJ/mol.

apparently also substantially interact with the neighboring flipped-in cytosine. In Table 5, we also calculated the relative potential energy changes corresponding to the $\Delta \Delta G$'s presented in Table 1. These values are all negative and in the range between -12.4 and -27.2 kJ/mol, which can explain the tendency of lower propensities for extra-helical conformations when either of the cytosines in the CpG site is methylated. Note that for these relative energy changes it seems sufficient to calculate only the interaction energy between the mutated cytosine and water. This suggests that the other interactions, such as those between the water molecules (which exhibit much larger errors), are less important to the processes under consideration and are likely to cancel out within the thermodynamic cycles.

The fact that the cytosines in unmethylated CpG sites exhibit larger propensities to adopt spontaneous extra-helical conformations compared with that of hemimethylated CpG sites indicates that this behavior does not correlate with the need in biological systems to flip out these cytosines. In hemimethylated CpG sites, both cytosines have been demonstrated to flip out into a binding pocket of a protein for either recognition or catalysis, whereas these extra-helical conformations are not detected, or known to be of biological significance, for unmethylated or fully methylated sites. Note that the reading of the fully methylated epigenetic marks by the methyl-CpG binding proteins, such as MeCP2, is performed while both cytosines are in their intra-helical conformations and no base flipping is observed.⁷⁶ Therefore, the results presented in this paper, although by no means can be considered as a prove, suggest that from efficiency arguments it is not likely that the mechanism for flipping out the cytosines in CpG sites is passive in which spontaneous base flipping precedes the binding to the protein.

IV. CONCLUSIONS

In this study, we calculated by alchemical mutations the relative free energy changes of flipping out the cytosine bases in unmethylated, hemimethylated, and fully methylated CpG sites. Analogous to experimental findings that involve methylation of N6 of adenine in GATC sites, we find that C5 methylation of cytosines in CpG sites reduces the propensities of the extra-helical states. Thus, the cytosines in unmethylated CpG sites are more likely to undergo spontaneous base flipping than the cytosines in hemimethylated sites and the latter are more likely to flip out than the cytosines in fully methylated sites. This reduction is found to be additive with respect to the degree of methylation. Methylation of one cytosine increases the free energy of the extra-helical conformation by $10.3\text{--}16.5 \pm 5.9$ kJ/mol. Furthermore, we constructed potentials of mean force for flipping out unmethylated and methylated cytosines in unmethylated and hemimethylated CpG sites. The shapes of these curves indicate that, although methyl-cytosine does not exhibit a preference for flipping via either of the grooves, unmethylated cytosine does prefer flipping via the major groove. These PMFs also indicate that the free energy change of flipping out unmethylated cytosine is 20.5 ± 6.7 kJ/mol relative to that of methyl-cytosine. We also performed alchemical mutations between unmethylated and hemimethylated CpG sites in which the transformations between the two states passed via an intermediate state. The conclusion from these cycles is that the dominant factor for the different relative free energy changes is not steric or hydrophobic but is the change in the partial charges of the cytosine ring upon methylation. Given the larger number of known complexes between proteins and hemimethylated CpG sites in which one of the cytosines is extra-helical (compared with CpG sites in other methylation states), we conjecture that the mechanism for flipping out the cytosines in these cases is not likely to be spontaneous (passive).

■ ASSOCIATED CONTENT

Supporting Information

Analyses of the widths of the major and minor grooves, the populations of the BI and BII states, and snapshots of a fully methylated DNA in an extra-helical conformation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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