Regioselective Effect of Zwitterionic DNA Substitutions on DNA Alkylation: Evidence for a Strong Side Chain Orientational Preference†

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ABSTRACT: The incorporation of zwitterionic residues (5-substituted ω-aminoalkyl-2′-deoxypyrimidines) into DNA has been reported to bend DNA as measured by aberrant gel mobility [Strauss et al. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93,* 9515−9520]. Herein we report that DNA methylation by *N*-methyl-*N*-nitrosourea at N7-guanine is regioselectively inhibited by point substitutions of the zwitterionic residues 5-(6-aminohexyl)-2′-deoxycytidine, 5-(6-aminohexyl)-2′-deoxyuridine, or 5-(3-aminopropyl)-2′-deoxyuridine. No inhibition is observed for DNA methylation by dimethyl sulfate. On the basis of inhibition patterns for methylation with the different zwitterionic substitutions and the different length tethers, the ω-aminoalkyl side chains prefer to adopt a conformation that points them toward the 3′-base. Molecular modeling grid searches, coupled with energy minimizations, and simulated annealing molecular dynamics studies indicate that unfavorable steric interactions with the 5′-base and backbone, as well as stabilizing electrostatic interactions with electronegative atoms on the 3′-side, are responsible for the observed conformational preference. No evidence for association of the cationic side chain with the phosphate backbone is observed. The observed bending of DNA induced by the tethered ammonium ions may simply arise from their localization in the major groove.

In addition to their biological effects, *e.g.,* mutations (Searle, 1984) and cytotoxicity (Pratt & Ruddon, 1979), alkylating agents have been used to probe the primary, secondary, and tertiary solution structure of DNA. For example, dimethyl sulfate (DMS),[1] which reacts with DNA primarily at the N7-G position (∼80% of total adduct yield) (Beranek et al., 1980), shows little selectivity in its reactions with duplex B-DNA, making it a useful reagent to sequence G (Maxam & Gilbert, 1980). However, the reactivity of DMS is sensitive to steric factors that affect the accessibility of N7-G, so it can be used to map dsDNA−protein and dsDNA−ssDNA complexes that involve major groove contacts (Mueller & Wold, 1991). In contrast to DMS, the potent carcinogen *N*-methyl-*N*-nitrosourea (MNU), which also predominantly reacts at N7-G (∼65% of total adduct yield), shows a significant sequence selectivity at G (Wurdeman & Gold, 1988). The yield of DNA adducts from MNU is actually enhanced in dsDNA *vs* ssDNA, so steric factors are not significant in its modification of DNA (Wurdeman et al., 1993). It has been argued that electrostatic influences (Wurdeman & Gold, 1988; Wurdeman et al., 1989, 1993; Pullman & Pullman, 1981; Zakrzewski, & Pullman, 1985; Rajalakshmi et al., 1978), as well as π polarization (Kim & LeBreton, 1996), are important for both the quantitative and qualitative reactions of MNU with DNA.

The role of electrostatic association between small charged alkylating species and DNA has been studied using several experimental approaches, including the employment of zwitterionic DNA substitutions, *i.e.,* pyrimidines modified with a 5-aminoalkyl side chain (Liang et al., 1995). The inhibitory effect of 5-(6-aminohexyl)-2′-deoxycytidine (Z6dC) zwitterionic substitutions on DNA methylation by MNU has been reported (Liang et al., 1995). It was shown that a Z6dC residue causes a regioselective inhibition of methylation at N7-G located 2−3 base pairs toward the 5′-terminus on the complementary strand: no effect was seen at G base paired to the zwitterionic residues or to G in the 3′-direction on the complementary strand. Moreover, no decrease in methylation was observed with the bulkier methylating agent, dimethyl sulfate (DMS). Quantitative analysis of major groove N7-methylguanine (N7-MeG) and minor groove N3-methyladenine (N3-MeA) adducts showed that zwitterionic residues selectively inhibit methylation in the major groove. This was anticipated since the aminoalkyl side chain of zwitterionic residues is tethered to the C5-position of the pyrimidine, which positions the cationic charge in the major groove. It was concluded that the zwitterionic residue inhibits an electrostatic interaction between the reactive

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[1] Abbreviations: DMS, dimethyl sulfate; MNU, *N*-methyl-*N*-nitrosourea; N3-MeA, N3-methyladenine; N7-MeG, N7-methylguanine; nBudC, 5-butyl-2′-deoxycytidine; VDW, van der Waals; Z3dC, 5-(3-aminopropyl)-2′-deoxycytidine; Z3dU, 5-(3-aminopropyl)-2′-deoxyuridine; Z6dC, 5-(6-aminohexyl)-2′-deoxycytidine; Z6dU, 5-(6-aminohexyl)-2′-deoxyuridine; zwitterionic residue, 5-(ω-aminoalkyl)-2′-deoxypyrimidine.

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intermediate generated from MNU, *i.e.*, methanediazonium ion ($CH_3N_2^+$), and the negative electrostatic potential in the major groove (Liang et al., 1995).

Elegant *in vitro* studies have demonstrated that the electrostatic environment of DNA may alter its structure. DNA bending (~21°) is observed when neutral methyl phosphonate linkages are appropriately phased with an intrinsically bent A-tract sequence (Strauss & Maher, 1994). More recently, Z6dC and Z3dU residues have also been used in an attempt to create neutral patches similar to the methyl phosphonates, and ~4° and 8° bending, respectively, is observed (Strauss et al., 1996a,b). It was proposed that the bending arises from the qualitative neutralization of charge on the phosphodiester backbone. For the zwitterionic residues, the neutralization of charge would result from the formation of a salt bridge between the alkyl-tethered ammonium ion of the side chain and a nonbonding phosphate oxygen (Strauss & Maher, 1994; Strauss et al., 1996a,b). In both cases, the result is suggested to be a local narrowing of the minor groove. A similar phenomenon may be responsible for the bending that occurs when basic amino acid side chains of proteins form salt bridges with the DNA phosphate backbone (Manning et al., 1987).

Because we have observed that zwitterionic residues inhibit DNA methylation with high regioselectivity that is not indicative of salt bridging with the 5′-phosphate (Liang et al., 1996), a more thorough analysis of the positioning of the aminoalkyl side chains in the major groove was undertaken. The results confirm the regioselective inhibition of N7-MeG formation by the zwitterionic residues and indicate that the aminoalkyl appendages on DNA do not sample conformational space but are severely restricted because of stereoelectronic interactions. No evidence for interaction with the phosphate backbone is observed.

EXPERIMENTAL PROCEDURES

Caution: Both DMS and MNU are toxic and potential human carcinogens and should be handled accordingly.

Materials. The zwitterionic phosphoramidite intermediates were synthesized and incorporated into deoxyoligonucleotides as previously described (Hashimoto & Nelson, 1993a,b). Solutions of MNU and DMS (Aldrich Chemical Co., Milwaukee, WI) were made up immediately before use and kept on ice.

DNA Methylation Studies. The oligomers were individually 5′-labeled with T4 kinase (Bethesda Research Labs, Gaithersburg, MD) in the presence of [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) and purified by electrophoresis using 12% polyacrylamide gels. The end-labeled DNA, with or without a 2-fold excess of the cold complementary strand, and cold sonicated calf thymus DNA (100 μM final concentration) were incubated with MNU at 4 °C in 10 mM sodium cacodylate buffer (pH 7.6) containing 50 mM NaCl for 16 h. Incubations with DMS (40 mM) were run in 15 mM sodium cacodylate buffer (pH 8.0) and 1 mM EDTA for 1 min at ambient temperature. Upon completion of the methylation reaction, the DNA was precipitated (NaOAc and EtOH), washed (cold 70% EtOH), and dried *in vacuo*. Strand breaks in the reacted DNA were generated by treatment with 1 M piperidine at 90 °C for 25 min to preferentially convert N7-MeG into strand breaks (Maxam & Gilbert, 1980). After removal of the piperidine *in vacuo*, the dried DNA was suspended in loading buffer (80% deionized formamide, 50 mM Tris−borate, pH 8.3, 1 mM

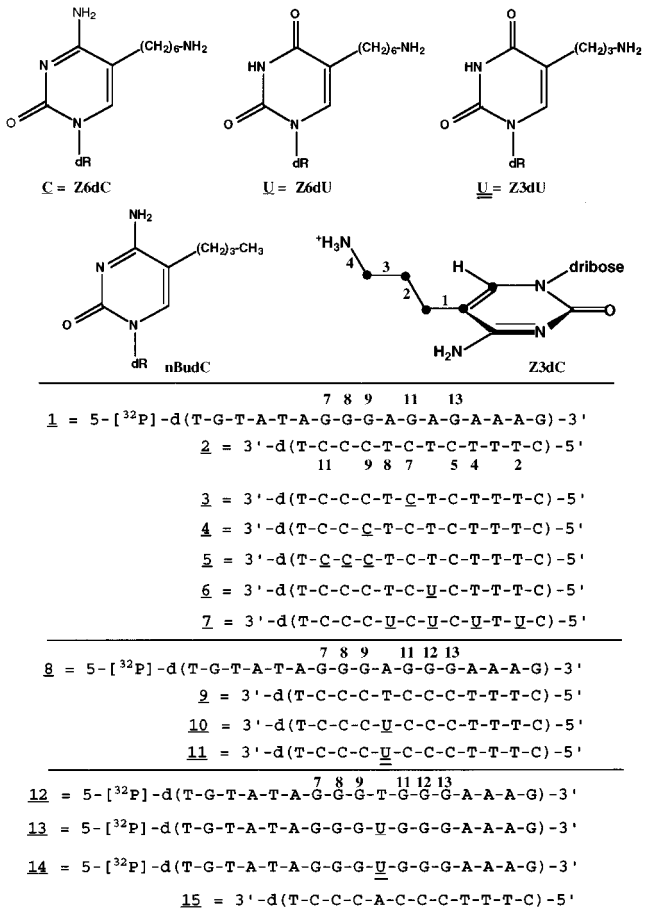

FIGURE 1: Structures of zwitterionic residues and oligomers: C = Z6dC; U = Z6dU; U = Z3dU.

EDTA, no dye markers) and denatured by heating at 90 °C for 1.5 min and cooling in ice. The DNA was electrophoresed using 20% polyacrylamide (7.8 M urea) denaturing gels run at 65 W. The standard G and/or G+A reaction lanes were included as sequence markers (Maxam & Gilbert, 1980). The gels were analyzed on a Molecular Dynamics PhosphorImager (Sunnyvale, CA). The phosphorimaging data were quantitated and then normalized to an assigned band for each lane (see figure legends). This normalization corrects for any differences in loading between the different lanes on the same gel.

CD Studies. CD spectra of 20 μM (nucleotide) **1** + **2** and **1** + **7** in 10 mM sodium cacodylate buffer (pH 7.3) containing 50 mM NaCl were obtained at ambient temperature on a Jasco 710 CD spectrophotometer.

Molecular Modeling Studies. Duplex B-DNA 5′-d(CTT-TCC-Z3dC-CCCCT)·3′-d(GAAAGGGGGGGA) was constructed using the Arnott coordinates (Arnott & Hukins, 1972) found in the Biopolymer building routine in Sybyl (Tripos Associates, St. Louis, MO). A 3-aminopropyl side chain was appended onto the 5-position of C-7. Charges for the 3-aminopropyl side chain used in the minimizations were derived from the Kollman United Atom side chain charges used for lysine (Weiner et al., 1984). The conformational preference of the side chain was investigated by two methods: grid search, coupled with energy minimization, and simulated annealing molecular dynamics.

In the grid search approach, the four rotatable bonds of the 3-aminopropyl side chain (Figure 1, Z3dC) were systematically scanned at 60° intervals from 0 to 300°. After each rotation, which generates a new conformer, the molecule

was minimized using the Kollman United Atom force field for 200 iterations, and the structure was saved. During the minimization, the DNA (not including the side chain) was treated as an aggregate so that the duplex DNA structure was not perturbed. In the initial studies, the dielectric constant used in the calculations was set to either 4 or a distance-dependent dielectric $\epsilon = R_{i,j}$. In addition, the charges on the two nonbonding phosphate oxygens (Kollman atom type O2) (Weiner et al., 1984) were set to either −0.20 or −0.85 during the analysis. There was no change in the overall hierarchy of conformers using the different dielectric treatments or phosphate oxygen charges, so all subsequent grid search studies incorporated a phosphate nonbonding oxygen charge of −0.20 and a distance-dependent dielectric.

Upon completion of the grid search analysis, the structures were sorted by energy. The ten lowest energy conformations for each 60° torsional interval for torsion 1 (defined in Figure 1) were selected and minimized using the Kollman United Atom force field until ΔG was ≤ 0.03 kcal·mol^{-1}. The relationships between torsion 1 and total, van der Waals (VDW), and electrostatic energies were plotted. This process was repeated for all 16 possible combinations of bases (5′-N-Z3dC-N-3′) flanking the zwitterionic residue. A similar analysis was performed with a *n*-butyl neutral side chain, *i.e.*, 5-butyl-dC (nBudC), using the grid search approach described above.

The conformational preferences of the aminopropyl, aminohexyl, and *n*-butyl side chains in C-Z3dC-C, C-Z6dC-C, and nBudC, respectively, were also analyzed using molecular dynamics simulated annealing and the Kollman United Atom force field with Kollman charges (Weiner et al., 1984). The analyses were done using two starting structures. In one structure the ammonium group was pointing out from the major groove so that the side chain is essentially perpendicular to the long axis of DNA. This starting conformation is defined as "unbiased". In the second starting structure for the dynamic studies, the ammonium ion was positioned approximately 2 Å from the major groove nonbonding phosphate oxygen that is immediately 5′ of the zwitterionic residue. This starting structure is defined as "biased". Depending on the structure, it was found that different "initial" temperatures were required to allow the side chains to sample conformational space. For this reason there is a variance in the initial temperature used for the different side chains. It was also determined for the Z3dC side chain that the charge on the nonbonded phosphate oxygens could be changed from −0.85 to −0.2 without any change in the hierarchy of the preferred structures. In all subsequent dynamic calculations with the Z6dC and nBudC side chains, the charge was set at −0.2. The following parameters were generally used in the dynamic runs: number of cycles, 10; time increment in history file, 50 fs; time increment for dynamics computation, 0.5 fs; coupling time for temperature regulation, 2.0 fs; equilibration time at initial temperature, 1500 fs; target temperature for annealing, 300 K; annealing time, 1500 fs; annealing function, exponential. The parameters that varied for the different side chains are as follows. Z3dC (biased): initial temperature, 2000 K; charge on phosphate oxygen, −0.85 or −0.2. Z3dC (unbiased): initial temperature, 1000 K; charge on phosphate oxygen, −0.85 or −0.2. Z6dC (biased and unbiased): initial temperature, 700 K; charge on phosphate oxygen, −0.2. nBudC (unbiased): initial temperature, 1000 K; charge on phosphate oxygen, −0.2.

RESULTS

Methylation Studies. The structures of the oligomers and the different zwitterionic residues used in the methylation studies are shown in Figure 1. The reaction conditions used were designed to ensure that the DNA substrate remains as a duplex throughout the reaction: the T_m's of **1** + **2**, **1** + **3**, and **1** + **4** are 39.4, 42.8, and 45.4 °C, respectively, in 10 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl (Hashimoto & Nelson, 1993a,b). Similarly, the stability of a dA_{12}·dT_{12} duplex (T_m = 22.5 °C) is not significantly affected by the introduction of a single or multiple Z3dC (T_m = 22.0 °C) or Z6dU (T_m = 22.5 °C) residues (Hashimoto & Nelson, 1993a,b). The 5′-overhang on the labeled strand (oligomer **1**, **12**, **13**, or **14**) is designed to allow detection of strand breaks at G that are very near the 5′-label and difficult to detect if perfect duplexes are used.

The methylation patterns at N7-G were revealed using Maxam−Gilbert G-lane chemistry, *i.e.*, direct treatment with hot piperidine (Maxam & Gilbert, 1980). The incubation conditions (concentration of methylating agent, reaction temperature, and incubation time) were determined to afford <1 N7-MeG lesion per strand. Under these conditions the intensities of the bands from MNU and DMS reflect the rates of reaction at the different G residues. The N7-G methylation profile generated with "normal" dsDNA [5′-^{32}P] **1** + **2** (Figure 2) shows the sequence selectivity that is characteristic of MNU (Wurdeman & Gold, 1988; Wurdeman et al., 1989). Specifically, the central G (G-8) in a G_3 run is most reactive. In contrast, DMS affords a uniform cleavage pattern at all G.

From previous studies using the same DNA target, the introduction of a single Z6dC residue ([5′-^{32}P] **1** + **3**) results in the selective inhibition of N7-MeG formation at G-8 and G-9, with no change at G-7, G-11, or G-13 (Liang et al., 1995). Multiple substitutions of Z6dC at C-5, C-7, C-9, and C-11 result in strong inhibition of methylation at all G except G-13. When duplex [5′-^{32}P] **1** + **5** is treated with MNU, only the bands corresponding to G-8 and G-7 are diminished (Figure 2). These G are all located 5′ of the zwitterionic residue on the complementary strand and are not directly across from the modification. As previously noted, the zwitterionic substitution(s) does (do) not have any effect on N7-G methylation by DMS (Liang et al., 1995).

The replacement of T-6 in oligomer **2** with a Z6dU residue ([5′-^{32}P] **1** + **6**) does not inhibit methylation by MNU (Figure 3A). The bands are somewhat darker for [5′-^{32}P] **1** + **6** relative to the normal DNA ([5′-^{32}P] **1** + **2**); however, normalization of the bands relative to G13 shows that this increase reflects a difference in loading (Figure 3B). In contrast, multiple substitutions of Z6dU for T ([5′-^{32}P] **1** + **7**) cause a significant reduction in methylation at all G (Figure 3). A comparison of Z6dU ([5′-^{32}P] **8** + **10**) and Z3dU ([5′-^{32}P] **8** + **11**) with normal DNA ([5′-^{32}P] **8** + **9**) shows that both residues produce a decrease in methylation at G-8 and G-9 (Figure 4). However, only the Z6dU substitution causes a significant diminution at G-7 (Figure 4B). It should be noted that the difference in alkyl chain length between Z3- and Z6dC is approximately 4.5 Å, which translates to a 1 base pair rise in the DNA helix if the side chain lays near the floor of the major groove.

To probe the inhibitory effect at G in the same strand as the Z6dU or Z3dU substitutions, the reactions of MNU with [5′-^{32}P] **13** + **15** and [5′-^{32}P] **14** + **15** were investigated

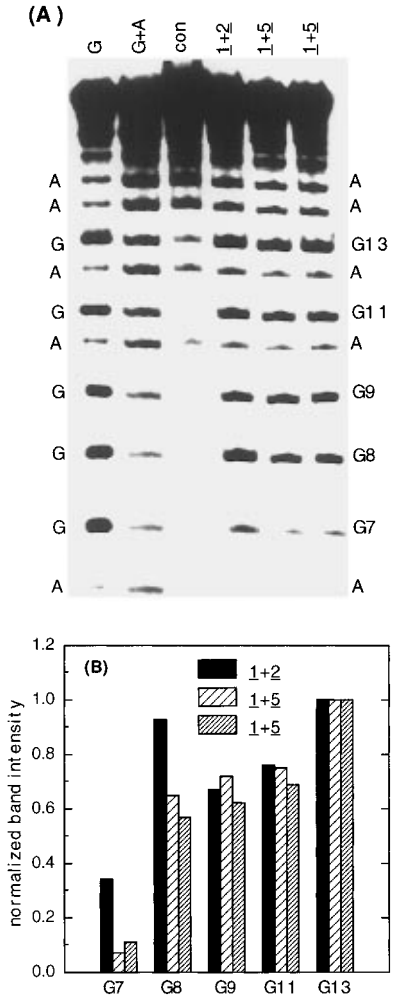

FIGURE 2: Reaction of 500 μM MNU with normal DNA and DNA containing zwitterionic Z6dC residues. (A) Phosphorimaging results of a denaturing 20% polyacrylamide gel using [5-^{32}P]-**1** with complementary strand **2** or **5** (run in duplicate). (B) Densitometry analysis of the gel. Band intensities in each lane are normalized to G13.

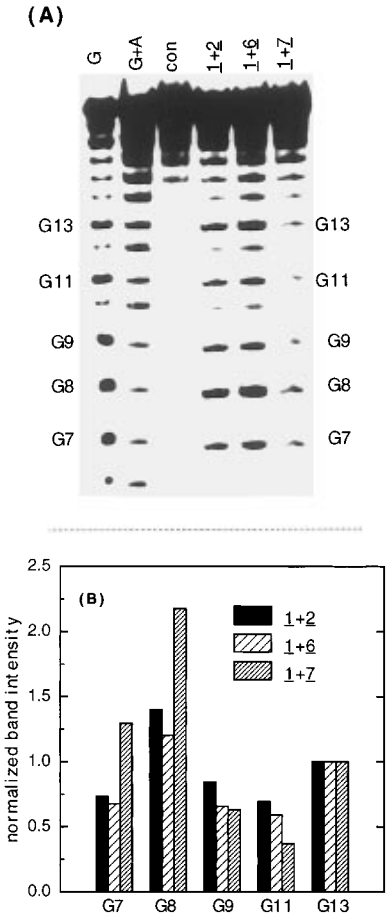

FIGURE 3: Reaction of 500 μM MNU with normal DNA and DNA containing Z6dU residue(s). (A) Phosphorimaging results of a denaturing 20% polyacrylamide gel using [5-^{32}P]-**1** with complementary strand **2** , **6** (Z6dU residue), or **7** (4 $\times$ Z6dU residues). (B) Densitometry analysis of the gel. Band intensities in each lane are normalized to G13.

(Figure 5). A band corresponding to the site of the Z3dU for T-10 substitution in oligomer [5′-^{32}P] **12** is apparent in the control (treated with hot piperidine but not MNU) and in all the other lanes containing oligomer **14**. This band only appears when the DNA is treated with hot piperidine. Heating the DNA without piperidine does not yield a strand break at this position; therefore, the band is attributed to a contaminating abasic site in oligomer **14**. Quantitative analysis of the control lane for oligomer **14** (no MNU with hot piperidine treatment) in Figure 5 provides an estimate that the abasic impurity is approximately 2−3%. We believe that during the hydrogenation of the triple bond in the course of the synthesis of Z3dU deoxynucleoside there is 1−2% overhydrogenation to produce the 5,6-dihydrouridine derivative of Z3dU. We have observed this side product previously, and it has virtually the same chromatographic properties as the uridine derivative. This 1−2% of the dihydrouracil analogue is carried through the oligomer synthesis, and upon deprotection with concentrated ammonia, the dihydrouridine ring is opened. The rate of depyrimidination of the dihydrouracil analogue is significantly more rapid than that of dU (House & Miller, 1996). The ring-opened dihydrouracil is cleaved off the sugar, and the resulting "abasic" site is converted into a strand break during the hot piperidine treatment used to map the N7-MeG sites. The presence of this abasic site at a level of a few percent will not have a significant impact on the data.

There is a decrease in the electrophoretic mobility of the Maxam−Gilbert G+A bands associated with G-11, G-12, and G-13 in oligomers **13** and **14** due to the positive charge on the Z-dU residues (Figure 5) ((Hashimoto & Nelson, 1993a,b). As expected, the same mobility shift is observed in the MNU-treated DNA. A comparison of normal DNA [5′-^{32}P] **12** + **15** with [5′-^{32}P] **13** + **15** shows that there is a significant decrease in methylation at G-11 through G-13 as a result of the Z6dU residue. No change is observed at the G toward the 5′-terminus of the labeled strand. A similar regioselective effect is observed with [5′-^{32}P] **14** + **15**. Quantitation of the bands at G-11 through G-13 suggests that the 6-aminohexyl side chain has a more pronounced inhibitory effect at the more distant G-13 residue than the 3-aminopropyl side chain; however, the difference is small and may not be significant.

In summary, zwitterionic substitutions cause a regioselective inhibition in DNA methylation by MNU. When the zwitterionic residue and the G are on opposite strands, the inhibitory effect is observed at G located toward the 5′-terminus on the complementary strand relative to the site of the zwitterionic substitution (Figure 6). When the G and the zwitterionic residue are on the same strand, the inhibition is toward the 3′-terminus.

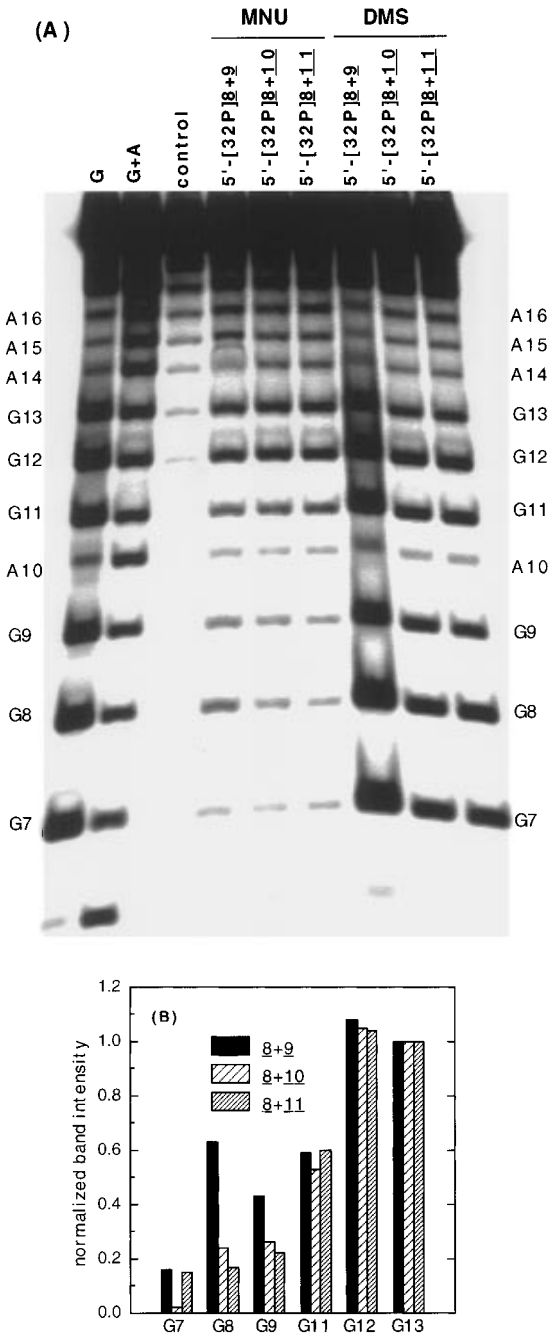

FIGURE 4: Reaction of 500 μM MNU or 40 mM DMS with normal DNA and DNA containing a Z6dU or Z3dU residue. (A) Phosphorimaging results of a denaturing 20% polyacrylamide gel using [5-^{32}P]-**8** with complementary strand **9**, **10** (Z6dU residue), or **11** (Z3dU residue). (B) Densitometry analysis of the gel. Band intensities in each lane are normalized to G13.

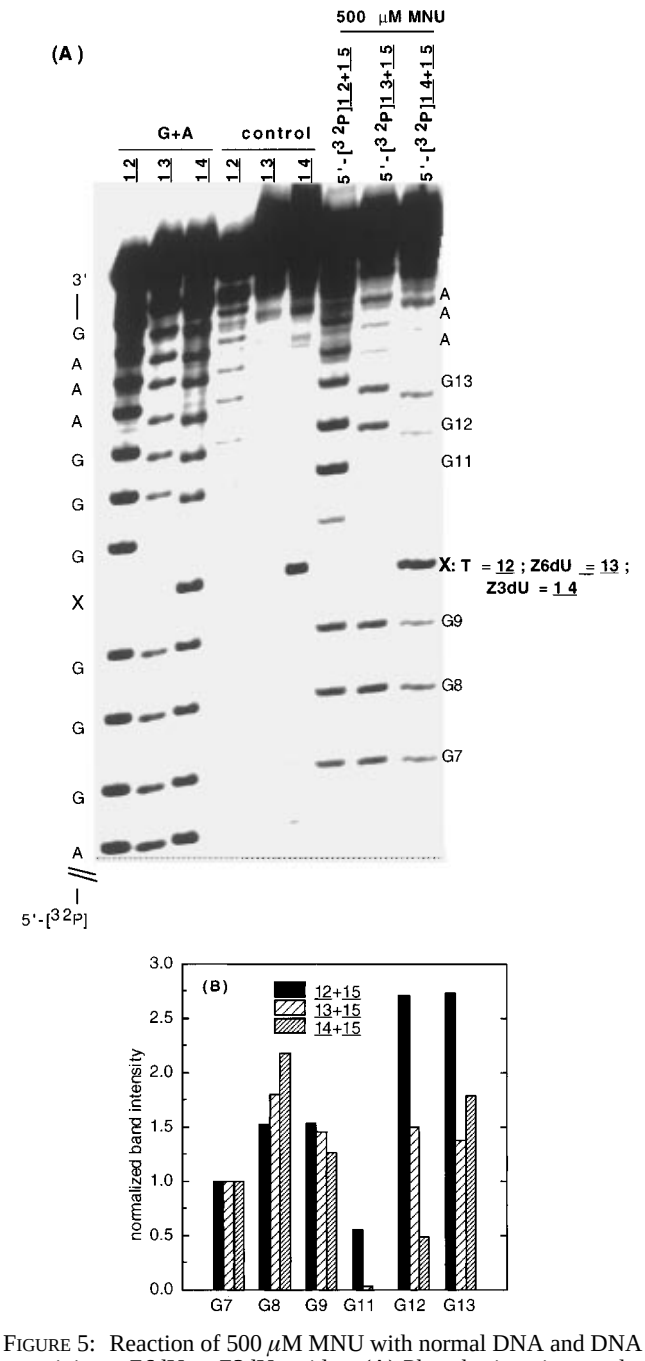

FIGURE 5: Reaction of 500 μM MNU with normal DNA and DNA containing a Z6dU or Z3dU residue. (A) Phosphorimaging results of a denaturing 20% polyacrylamide gel using [5-^{32}P]-**12**−**14** containing dT, Z6dU, and Z3dU, respectively, with complementary strand **15**. (B) Densitometry analysis of the gel. Band intensities in each lane are normalized to G7.

CD Studies. The possibility that the tethered cations could induce conformational changes in the dsDNA was explored using CD spectroscopy because of previous reports that G/C-rich sequences can undergo conformational changes upon the addition of specific cations (Laudon & Griffith, 1987; Braunlin & Xu, 1992; Brukner et al., 1994; Dlakic & Harrington, 1995; Robinson & Wang, 1996). The CD of a Z3dU-modified dsDNA that is based on **1** + **7**, but lacking the 5′-overhang on **1**, shows a clear B-DNA spectrum (data not shown). This spectrum is identical to that of the unmodified duplex (**1** + **2**). The results indicate that there is no gross global change in DNA conformation as a result of introducing the aminoalkyl side chains. The CD results do not address the possible presence of more subtle and/or local conformational changes induced by the tethered ammonium ions.

Modeling Studies. On the the basis of methylation data, the orientation of the aminoalkyl side chain is highly restricted (Figure 6). To understand the origin of this effect, a dsDNA with a Z3dC residue was modeled using both grid search and molecular dynamics. The energy of the potential side chain conformations in a B-DNA duplex was investigated using a grid search routine that allows a sampling of conformation space. In these studies the DNA was maintained as a rigid aggregate to make the computational analysis feasible. The four side chain torsional angles of the aminopropyl group were scanned at 60° intervals with 200 steps of minimization after each torsional rotation. A total

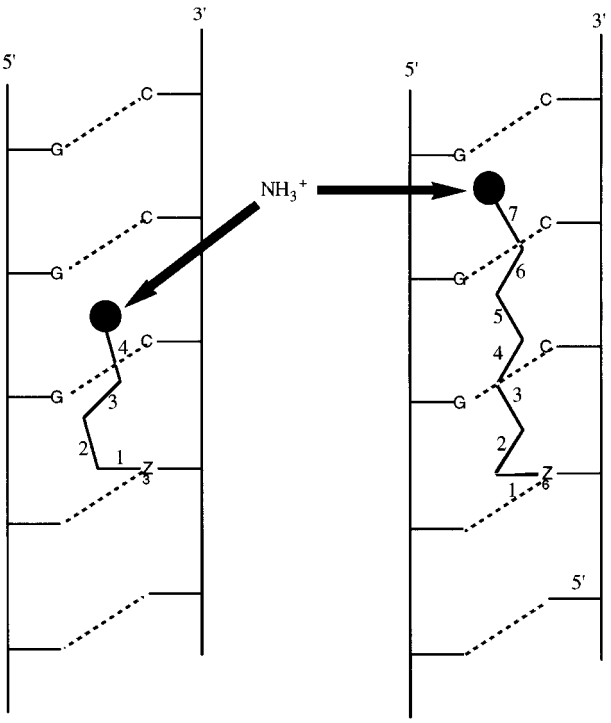

FIGURE 6: Proposed model for the orientational preference of zwitterionic 3-aminopropyl (left) and 6-aminohexyl (right) side chains.

of 1296 conformations were generated and minimized using the Kollman force field and Kollman United Atom charges (Weiner et al., 1984). At the conclusion of the analysis, the ten lowest energy structures were selected for each 60° torsional starting point, and these structures were minimized to convergence (<0.03 kcal·mol^{-1}). This process was repeated for all 16 possible arrangements of bases flanking the zwitterionic residue. The results of these studies for C-Z3dC-C are shown in Figure 7a and are very representative of the other sequences (data not shown). Some of the initial minimizations were done with different charges on the phosphate nonbonding oxygens (−0.2 and −0.85) and with different dielectric constants. However, neither phosphate charge nor dielectric constant affected the hierarchy of structures.

The lowest energy structures for all 16 sequences are clustered around a torsion 1 (Figure 1) value of −70°, and in most cases there is another low-energy cluster near −20°. These structures are derived from grid search torsion 1 angles of −60 and 0°, respectively. In both clusters, the side chain points to the 3′-side, and the electrostatic term dominates the energy differences between the conformers (Figure 7a). The VDW energies for the low-energy structures are actually slightly higher for the favored structures, but this is more than made up for in the electrostatic term. Surprisingly, no interaction is observed between the tethered ammonium ion and the phosphate backbone using a phosphate nonbonding oxygen charge of −0.85 (the charge normally assigned in the Kollman United Atom force field) or −0.2, or when the dielectric function is altered. The distance between the NH_3^+ nitrogen and the O^6-G for Z3dC is 2.86 Å (Figure 8a).

Identical results were obtained using simulated annealing to sample the conformational space of the Z3dC and Z6dC side chains in a sequence flanked by C: the low-energy structures point to the 3′-base, and there is the potential for electrostatic interactions with electronegative atoms in the major groove. In this case the distance between the NH_3^+ nitrogen and the O^6-G (Figure 8a,b) is 2.78 and 2.45 Å, respectively, for Z3- and Z6-dC. A comparison of the structure isolated from the dynamic run and the low-energy structures from the grid search for Z3dC shows almost a perfect match (Figure 8a). It should be noted that in both modeling approaches the DNA was maintained as a rigid aggregate. As part of the dynamic studies to see if a stable salt bridge structure would be generated, at the start of the run the ammonium ion was deliberately positioned approximately 2 Å from the nonbonded phosphate oxygen that points into the major groove. However, employment of this biased starting structure did not affect the outcome. In addition, the charge on the nonbonded phosphate oxygens was set at either −0.85 or −0.2, but no difference in the hierarchy of structures was seen in the dynamic runs.

The grid search and molecular dynamics treatments were applied to the same DNA with a neutral *n*-butyl side chain (nBudC, Figure 1), and similar results are obtained from both methods. The same orientational preference observed with the charged side chains is apparent (Figure 8c). Importantly, for the *n*-butyl side chain, both the VDW and electrostatic energies qualitatively correlate with the preferred low-energy structures (Figure 7b). The distance between the terminal CH_3 on the side chain and the 3′- O^6-G in the low-energy structure derived from molecular dynamics is 4.16 Å. This distance compares to the value of 5.03 Å that was obtained by grid search analysis (Figure 8c).

DISCUSSION

DNA Methylation. MNU hydrolyses to $CH_3N_2^+$, and it is this species that is responsible for the formation of methyl adducts (Wurdeman et al., 1989; Kriek & Emmelot, 1964; Kirmse, 1976; Gold & Linder, 1979; Smith et al., 1985; Koepke et al., 1984). Increasing the ionic strength of the reaction quantitatively inhibits DNA methylation by MNU and other alkylating agents that react via positively charged intermediates (Wurdeman & Gold, 1988; Rajalakshmi et al., 1978; Briscoe & Cotter, 1985; Hartley et al., 1990). DMS is not similarly affected (Wurdeman & Gold, 1988). Moreover, the ability of a cation to inhibit methylation by MNU correlates with the affinity of the cation for DNA: ethidium bromide > distamycin > Mg^{2+} > Na^+ (Wurdeman & Gold, 1988). Despite their quantitative effect, these cations do not alter the sequence specificity of MNU (Wurdeman & Gold, 1988). The sequence specificity of MNU is quite similar to that of other small positively charged alkylating agents (Hartley et al., 1986, 1988, 1990), and salt concentration only impacts on the overall yield, not the relative yield, of adducts (*e.g.*, N7-MeG *vs* N3-MeA) or the sequence-specific reactions with DNA. Therefore, it is implied that the sequence specificity of MNU is a function of local DNA stereoelectronic characteristics.

The gel data previously reported (Liang et al., 1995), and those presented here, demonstrate that the zwitterionic residues with their tethered ammonium ions can sequence selectively inhibit DNA methylation by MNU but not by DMS. The exact mechanism responsible for the inhibition by the zwitterionic residues is not certain, although it may involve the neutralization of the electronegative potential in the major groove (Gold & Wurdeman, 1988; Wurdeman et al., 1993; Pullman & Pullman, 1981) and/or an electrostatic repulsion between the tethered ammonium ion and $CH_3N_2^+$. What is certain is that the inhibition is not a result of steric

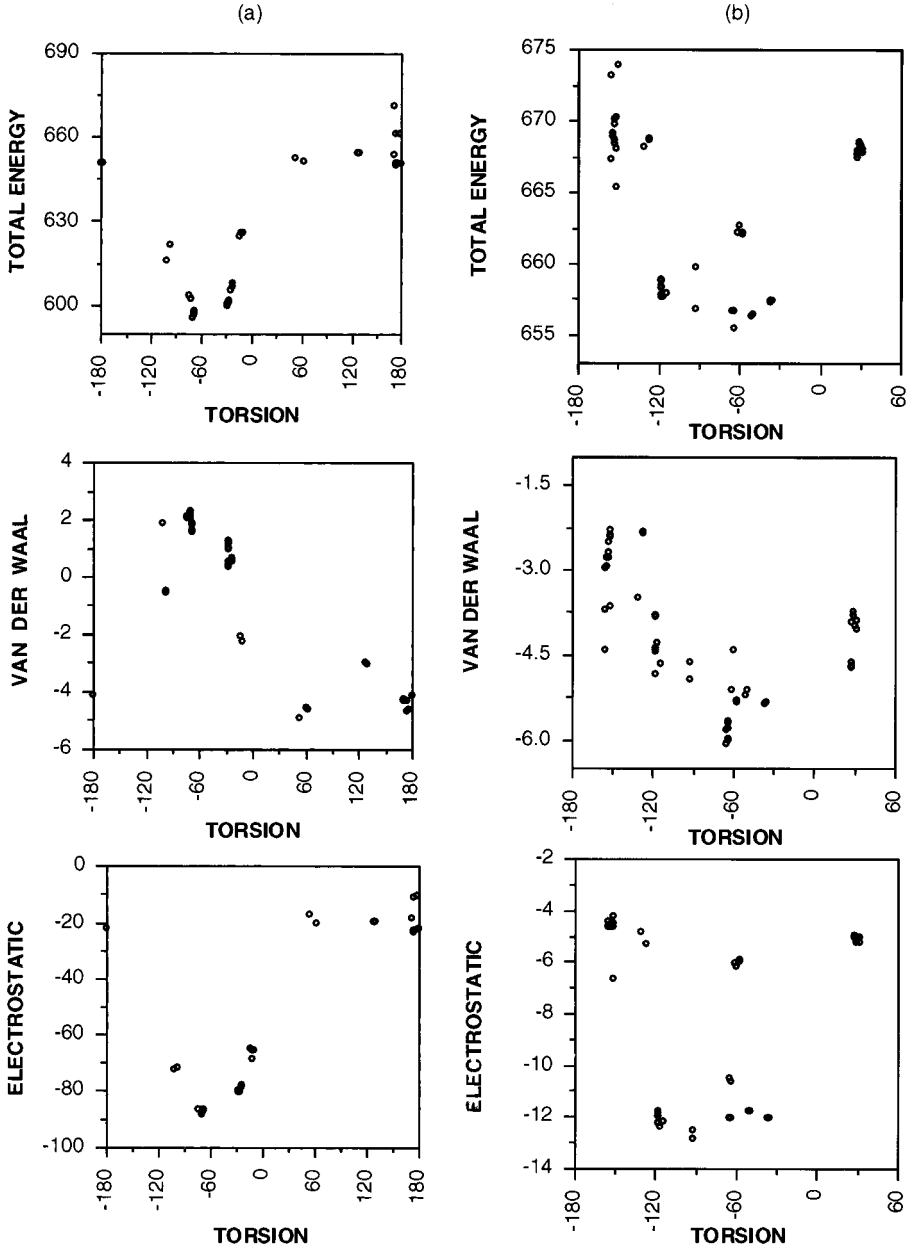

FIGURE 7: Molecular modeling grid search data: total, van der Waals (VDW), and electrostatic energies (kilocalories per mole) *vs* torsion 1 for (a) 5′-C-Z3dC-C and (b) 5′-C-nBudC-C.

inhibition by the aminoalkyl side chain since DNA methylation by the bulkier DMS is not affected by the presence of zwitterionic residues. On the basis of limited data, the side chain orientation toward the 3′-base was previously proposed (Liang et al., 1995), and the methylation data presented herein with Z6dC-, Z3dU-, and Z6dU-modified DNA unconditionally support this orientational preference. From the inhibition patterns, it appears that the effective "reach" of the side chain, in terms of inhibiting DNA methylation, is approximately 3 base pairs for the 6-aminohexyl compounds and slightly less for the 3-aminopropyl appendage. The inhibition pattern, which is observed on both strands, is not consistent with the originally postulated interaction of the aminoalkyl side chain with the 5′-phosphate of the modified nucleotide (Hashimoto & Nelson, 1993a,b).

Modeling. The modeling of the side chain in B-DNA using the grid search approach reveals a common theme for all 16 5′-*N*-Z3dC-N sequences: in the low-energy structures the ammonium ion points toward the 3′-base and is in close proximity to the electrophilic atoms in the major groove. This conformation minimizes steric interactions with the 5′-base and side chain, while allowing maximum electrostatic stabilization with the 3′-bases. The electrostatic interaction is the largest contributing factor to the stability of the 3′-orientation with torsion 1 having an energy minimum near −70°. This is also the only orientation that shows any H-bond stabilization, albeit small (<0.5 kcal·mol^{-1}). The electrostatic effect does not involve the phosphate backbone. This is the case using a variety of parameters, including −0.2 and −0.85 charge on the phosphate nonbonding oxygens and the use of a constant or distance-dependent dielectric. The former value for the phosphate charge is based on the effective screening of phosphate charge in the presence of 50 mM NaCl (Manning, 1978; Manning et al., 1989), the salt concentration used in the DNA methylation studies. Conformations that direct the side chain in the 5′-direction must avoid steric clash with the 5′-base and backbone. This can only occur if the side chain points out into the major groove, thus preventing any favorable electrostatic interaction between the ammonium ion and DNA. The same results

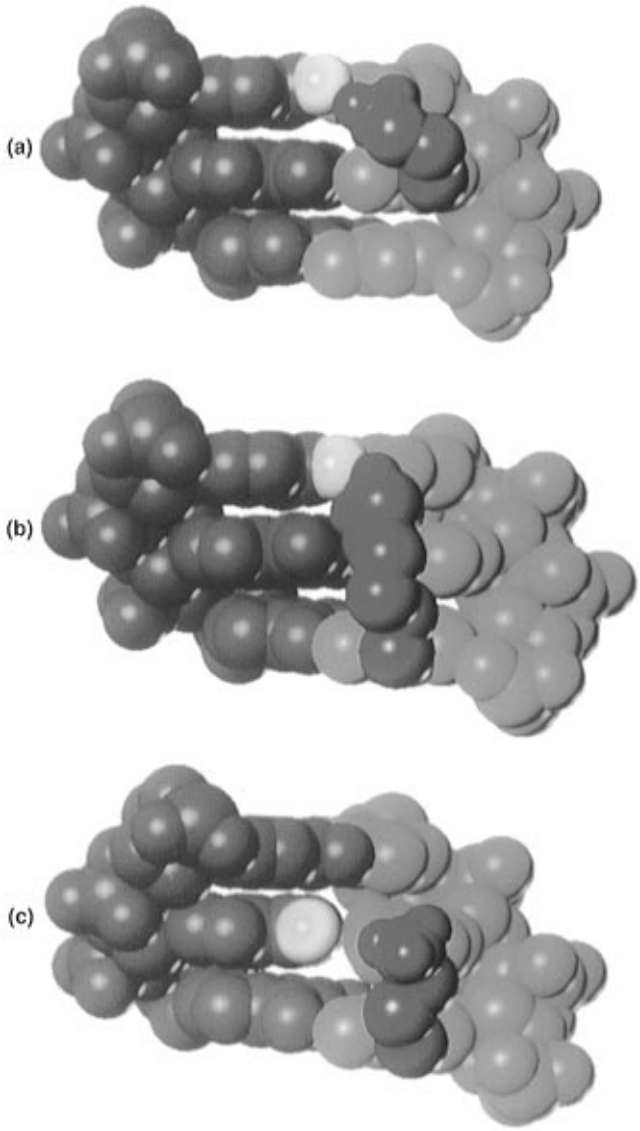

FIGURE 8: CPK structures derived from molecular modeling of modified residues in 5′-d(CTTTCC-**X**-CCCCT)·3′-d(GAAAGG-GGGGGA); **X** = Z3dC, Z6dC, and nBudC. The following color pattern applies to all three structures: green = pyrimidine strand; blue = purine strand; red = zwitterion side chain; and yellow = O^6-position of G. The DNA residues not associated with the side chain have been deleted for clarity. Panels: (a) overlap of Z3dC minimum energy structures derived from grid search and molecular dynamics simulated annealing; (b) Z6dC minimum energy structure from molecular dynamics simulated annealing; and (c) nBudC minimum energy structure from molecular dynamics simulated annealing.

are observed using simulated annealing, including runs in which the ammonium ion is initially placed in close proximity to the 5′-phosphate nonbonded oxygens.

In order to further dissect the electrostatic and steric factors involved in the side chain orientation, modeling was done using a neutral *n*-butyl in place of the 3-aminopropyl side chain. The 3′-orientation is still favored. The distance between the terminal methyl group on the *n*-butyl side chain in nBudC and the electronegative O^6-G position is 5.03 Å for the low-energy structure identified by the grid search approach (Figure 8c) and 4.16 Å from molecular dynamics. These distances are significantly greater than that seen with the charged side chains: 2.86 (Z6dC) and 2.45 Å (Z3dC), respectively. The results support the argument that the 3′-orientation is, in part, preferred because of steric interactions with the 5′-nucleotide.

In summary, torsion 1 is driven so that the side chain points to the less hindered face of the base. In addition, this orientation allows the interaction of the ammonium ion with the electronegative atoms in the major groove but not with the phosphate backbone.

Generality of Side Chain Orientation. The conformational preference of the major groove side chains that we report for zwitterionic residues has been previously observed although not discussed in any detail. An elegant case is the orientational preference of DNA interstrand cross-links formed by nitrogen mustards (Ojwang et al., 1989; Grueneberg et al., 1991; Millard et al., 1990; Rink et al., 1993). In these studies it was demonstrated that 5′-GNC sequences were the major sites for N7-G to N7-G interstrand cross-linking. The mustard results are consistent with the initial formation of a monofunctional N^7-[2-(*N*-(2-chloroethyl)amino)ethyl]-G half-mustard adduct that points the ionized tertiary amine ($pK_a \sim 6.5$), or aziridinium ion intermediate, toward the 3′-base because of the same steric and electronic factors implicated in the positioning of the zwitterionic residues. This places the alkylating moiety of the half-mustard near the 5′-G on the complementary strand and allows for closing of the cross-link via reaction at N7-G. Another example of the orientational preference is formaldehyde N^6-dA-CH_2-N^6-dA interstrand cross-linking specifically at d(AT) and not d(TA) sequences (Huang et al., 1992). In fact, we can find *no* example of a major groove DNA cross-linking event that does not show the same orientational preference predicted from the zwitterionic results. The similar conformational preference for both cationic (mustard) and neutral (formaldehyde) side chains indicates the generality of the orientational phenomenon.

DNA Structure and Flexibility. It is clear from molecular models of B-DNA that the inhibitory effect of the zwitterionic residues on DNA methylation exceeds the physical length of the tether. In Z3dU (a four-atom bridge), the aminoalkyl side chain can physically extend approximately 4 Å through space (Figure 8a), but inhibition is seen 2−3 bases pairs away, which is equivalent to >8 Å in B-DNA. The Z6dU, with its seven-atom bridge, can extend ∼8 Å above the plane of the pyrimidine ring, yet it influences methylation 3 base pairs away, or >11 Å. It is unlikely that the zwitterionic residues induce a global change in DNA structure because the CD spectrum of **1** + **7** with four zwitterionic residues is identical to that of unmodified DNA (**1** + **2**), the T_m's are similar to Watson−Crick DNA (Hashimoto & Nelson, 1993a,b), the incorporation of zwitterionic residues into polymers does not significantly alter the helical repeat (Strauss et al., 1996a), and the methylation at G that is directly based paired to zwitterionic residues is not altered (Liang et al., 1995). We suggest two schemes to explain the apparent reach of the zwitterionic side chains: (1) DNA is more conformationally promiscuous than anticipated, and/or (2) the tethered cationic charge localized in the major groove induces a local deformation in DNA structure. Our data cannot distinguish between these possibilities, which are not mutually exclusive. In reference to the last possibility, it is important to note that zwitterionic residues, when phased with inherently bent A-tracts, cause increased aberrant gel mobilities (Strauss et al., 1996,b). These data are evidence that DNA bending is induced by the tethered cationic charges.

A similar distance incongruity exists for the structure of the predominant nitrogen mustard mechlorethamine inter-

strand cross-link. Mechlorethamine links N7-G on complementary strands at 5′-GNC that involves a five-atom bridge between the DNA strands. While it is conceivable that a unique conformation may be trapped in the second step of interstrand cross-link formation, it is difficult to understand why the 5′-GNC, which is modeled as a highly deformed structure (Rink & Hopkins, 1995), is the major product rather than the originally proposed 5′-GC cross-link (Brookes & Lawley, 1961). Clearly the physical distances in B-DNA are not consistent with the effectiveness of the zwitterionic residues to inhibit DNA methylation or the ability of mustards to cross-link DNA. Since zwitterionic residues can induce DNA bending (Strauss et al., 1996a,b), it is possible that the initial monofunctional N7-G adduct, which is a ionized amine, can induce a change in DNA conformation that facilitates the formation of the second linkage.

CONCLUSIONS

We have demonstrated that 5-(ω-aminoalkyl)-2′-deoxypyrimidines (zwitterionic derivatives of dC and dU) can be used to regioselectively inhibit DNA methylation at N7-G by $CH_3N_2^+$. Moreover, the inhibition pattern serves as a straightforward probe of the positioning of the aminoalkyl side chains in the major groove. The inhibition data and molecular modeling demonstrate that these side chains adopt very predictable orientations toward the 3′-base, a result due to a combination of steric and electrostatic interactions. This orientational preference must be taken into account when appendages are substituted onto DNA. The results also extend previous *in vitro* studies that indicate that DNA may be much more flexible than envisioned or that the localization of positive charge in the major groove may decrease the rigidity of DNA and permit DNA bending (Brukner et al., 1994).

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SUPPORTING INFORMATION AVAILABLE

Grid search/energy minimization of all 16 possible N-Z3dC-N: plot of torsion 1 *vs* total, electrostatic, VDW, and hydrogen bond energies (17 pages). Ordering information is given on any current masthead page.

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