Reviews

A Review of the Role of the Sequence-Dependent Electrostatic Landscape in DNA Alkylation Patterns

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Alkylating agents, including environmental and endogenous carcinogens and DNA targeting antineoplastic agents, that adduct DNA via intermediates with significant cationic charge show a sequence selectively in their covalent bonding to nucleobases. The resulting patterns of alkylation eventually contribute to the agent-dependent distributions and types of mutations. The origin of the regioselective modification of DNA by electrophiles has been attributed to steric and/or electronic factors, but attempts to mechanistically model and predict alkylation patterns have had limited success. In this review, we present data consistent with the role of the intrinsic sequence-dependent electrostatic landscape (SDEL) in DNA that modulates the equilibrium binding of cations and the bonding of reactive charged alkylating agents to atoms that line the floor of the major groove of DNA.

1. Introduction

Genomic DNA is considered thermodynamically and chemically stable. However, because of its enormous size (>3 × 10^9 base pairs) and the sensitivity of cells to even very low levels of DNA damage, the relatively inefficient chemical reactions between DNA and endogenous and exogenous compounds has significant biological relevance. Our comments here are directed toward alkylation reactions in which DNA serves as a nucleophile, a common theme for many mutagens, carcinogens, and/or antineoplastic agents (*1*, *2*). In most cases, electrophilic attack on DNA affords a complex mixture of lesions derived from reactions at different positions on the four nucleobases and the phosphate backbone (*3*, *4*). If not correctly repaired before DNA replication, many of the lesions can induce cellular toxicity and/or mutagenicity. The former is associated with chemotherapeutic drugs and the latter with agents, including the same chemotherapeutic drugs, involved in the initiation of cancer.

Mutation frequencies vary depending on DNA sequence and often concentrate at sites referred to as hotspots (*5*). The hotspots reflect a combination of factors starting with the level of modification at a particular site, that is, the lower the level of adduct, the lower the mutation frequency. This phenomenon is the basis for the dose−response between mutagen exposure and mutation levels and presumably cancer incidence and latency (*6*, *7*). If a base is modified, then the generation of a mutation will depend on the rate and accuracy of repair and the way the lesion is processed by DNA polymerase(s). Both repair and replication may also be sequence-dependent (*8*−*10*). These factors (level of damage, rate of accurate repair, and error rate in polymerization) provide the real mutation frequency at an individual site, but the apparent mutation frequency is dependent on the selection factors used to detect mutations in a particular assay. The bottom line is that the mutation frequency at an individual base follows a dose-dependent response to exposure to a mutagen using nonsaturating doses. This is the case for

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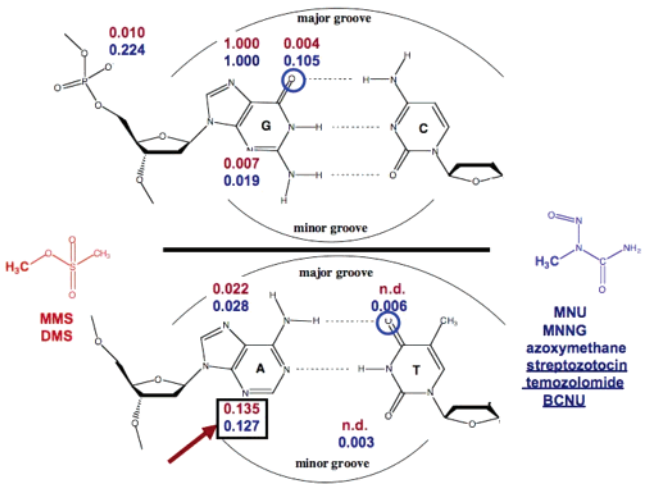

Figure 1. Methyl adducts produced from alkylating agents that react via cationic (blue) and neutral (red) intermediates. Yields are normalized relative to the predominant 7-mG lesion (based upon refs *3* and *4*).

alkylating agents, for example, *N*-methyl-*N*-nitrosourea (MNU[1]), which target 3′-G's in 5′-GG sequences (see below) and induce mutations with high frequency at 3′-G's in the 5′-GG sequences in all species. More complex DNA damaging molecules often show unique alkylation profiles (*11*), and there is also strong correlation between sites of covalent reaction of these complex electrophiles, for example, B[*a*]P-diol epoxide, and sites of mutations (*12*). The goal of our research is to understand the origin of sequence-dependent alkylation patterns.

2. Reactions of DNA with Simple Alkylating Agents

After it became apparent that covalent modification of DNA is an early critical step in mutagenesis and carcinogenesis (*13*, *14*), elegant work from many laboratories provided a detailed qualitative and quantitative picture of the lesions formed by a variety of DNA alkylating agents, including some that are, or were, used in the treatment of cancer. Some of these agents directly react with DNA, while others undergo either chemical or enzymatic changes to reveal the reactive electrophilic intermediate. For reasons that will become clear later, we divide the agents into those that react via intermediates that are neutral and those that react via intermediates with significant positive charge. Examples of the former class are alkyl halides, alkane sulfonates, epoxides, and so forth, which directly add to DNA via an S_N2 pathway. The latter group includes *N*-nitroso compounds (nitrosamines and nitrosoureas), triazenes, nitrogen mustards. It is thought that the reactive intermediates generated from these compounds also react with DNA via a concerted bimolecular pathway (*15*, *16*).

The types of lesions formed by methylating agents from the two classes, neutral and cationic, are shown in Figure 1 (*3*, *4*). The yields are qualitatively and quantitatively similar, but there are some important differences. For both classes of alkylating agents, the major product forms at N7-G in the major groove. Alkylation levels at other ring nitrogens are comparable. The main difference is the level of attack at the exocyclic oxygens, for example, O^6-G, O^4-T, and the nonbridging phosphate oxygens (*3*, *4*). From a chemical yield standpoint, the differences

[1]Abbreviations: DDD, Drew−Dickerson dodecamer 5′-CGCGAATTCGCG′; DDD-1, 5′-CGCGAATXCGCG (X = 5-[3-aminopropyl]-2′-deoxyuridine]; DDD-2, 5′-CGCGAAXTCGCG; DDD-3, 5′-CGCGAAXXCGCG; DMS, dimethyl sulfate; ds-DNA, double-strand DNA; 7-mG, N7-methylguanine; 6-mG, O^6-methylguanine; MNU, *N*-methyl-*N*-nitrosourea.

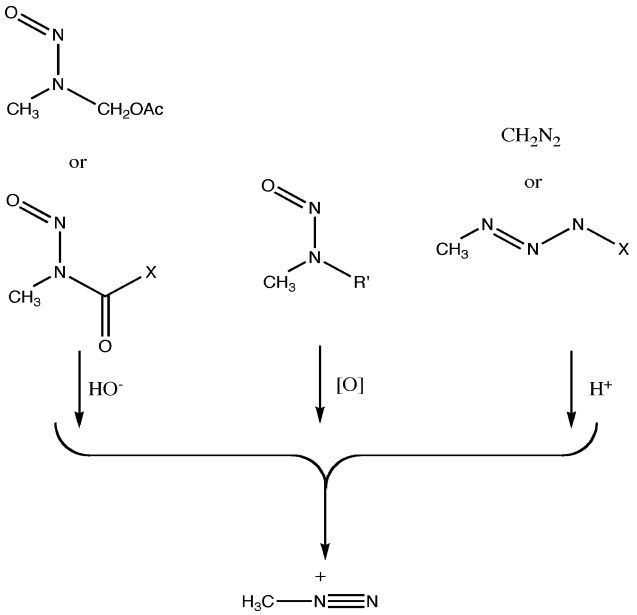

Figure 2. Conversion of α-acyl-*N*-nitrosamines, *N*,*N*-dialkylnitrosoamines and triazenes to a common methanediazonium ion.

are not large, but the biological consequences are significant as a result of the mispairing opposite some O-alkylated nucleobases by replicative or translesion polymerases and the resulting increase in the mutation frequency (*17*, *18*). It is important to note that the yields shown in Figure 1 are averages for genomic DNA and do not reflect yields at individual sites.

There are few reactions better understood than those of nucleophiles with alkyl sulfonate esters and halides. For reactions with ds-DNA, the issues of the accessibility and nucleophilicity of individual DNA sites need to be considered (*19*). On the basis of a combination of these two factors, the major product of ds-DNA alkylation occurs at N7-G. Obviously, sites that are reactive in nucleosides and ss-DNA become refractory to alkylation if they happen to be inaccessible upon formation of the Watson−Crick duplex. In general, yields at all positions are lower in ds-DNA than ss-DNA with the agents that react via noncharged intermediates due to steric factors.

A well-studied class of charged alkylating agents are the *N*-nitroso compounds, including nitrosamides, nitrosoureas, and nitrosamines. Through enzymatic or base-catalyzed hydrolysis or enzymatic α-oxidation (*20*, *21*), these compounds are converted into transient alkanediazonium ions (Figure 2) that provide the array of adducts shown in Figure 1 (*3*, *4*). For larger alkyl groups, for example, propyl or butyl, rearrangements via carbonium ion type intermediates are observed (*22*, *23*). Therefore, the array of products can become even more complicated because of the presence of structural isomers (*24*). Unsymmetrical *N*,*N*-dialkylnitrosamines, which have two sets of α-H's, can be oxidized to give two different alkanediazonium ions that in turn can yield two sets of DNA adduct products. The formation of structural adduct isomers has been well documented for the tobacco specific nitrosamines that can methylate and 4-oxo-4-(3-pyridyl)butylate DNA (*25*).

3. Sequence-Selective Reactions of Alkylating Agents with DNA

In general, the quantitation of adducts involves the digestion of the DNA to the nucleotide or nucleoside level, and this obliterates information concerning reactivity at specific sequences. In the first studies that provided insight into this

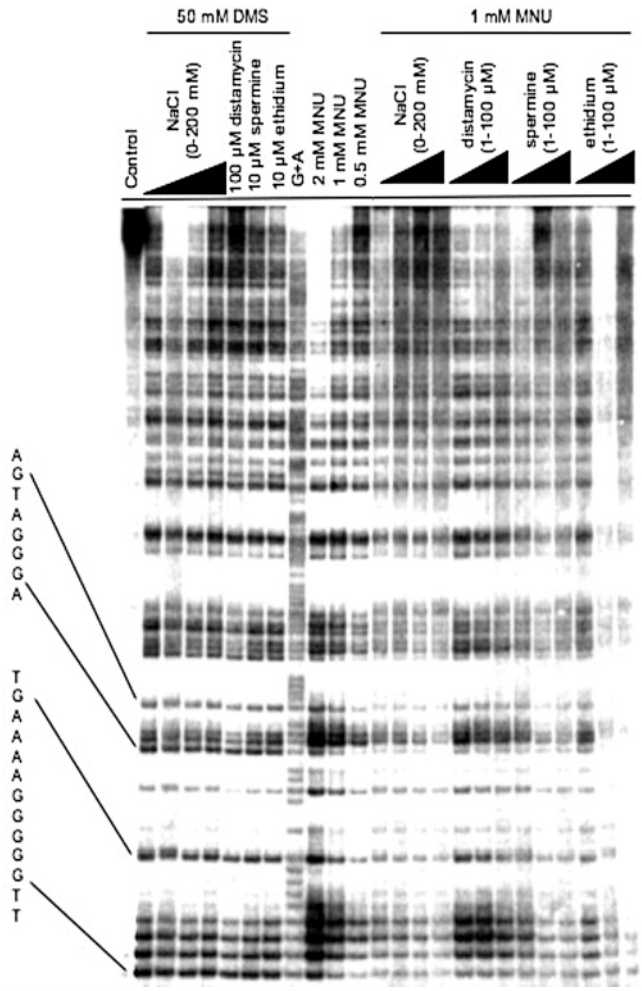

Figure 3. Methylation patterns at N7-G in an 85 bp restriction fragment produced from dimethyl sulfate (DMS) and *N*-methyl-*N*-nitrosourea (MNU) in 10 mM Tris-HCl buffer (pH 8.0) in the absence and presence of NaCl, distamycin, spermine, or ethidium (*32*).

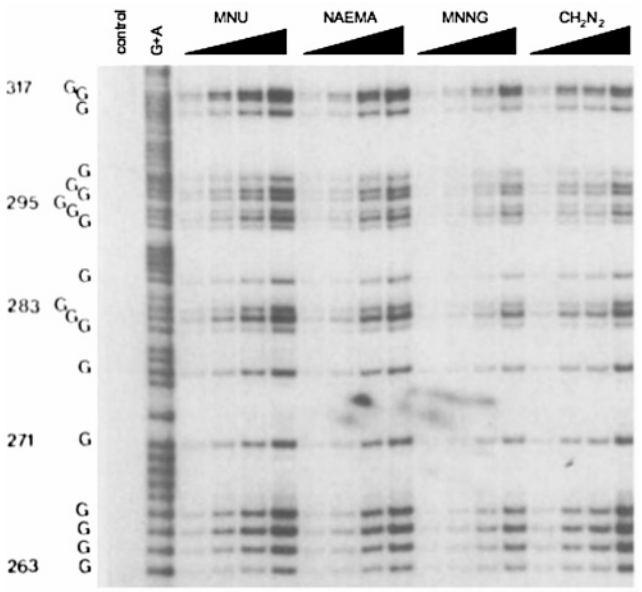

Figure 4. Methylation patterns at N7-G in the same sequence shown in Figure 3 produced from different concentrations of *N*-methyl-*N*-nitrosourea (MNU), *N*-nitroso(1-acetoxyethyl)methyl-amine (NAEMA), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), and diazomethane (CH_2N_2) (*32*).

question, it was shown that dimethyl sulfate (DMS) treated ds-DNA is uniformly methylated at all N7-G's (*26*) (Figure 3). The procedure involves the treatment of the methylated DNA with hot piperidine to (a) selectively open the *N*-methylimidazolium ring of N7-methylguanine (7-mG) by specific base-catalyzed hydrolysis; (b) remove the aminoformamidiopyrimidine from the DNA backbone by nucleophilic displacement; and (c) perform specific base-catalyzed hydrolysis of the resulting abasic site to create a single strand break (*27*). The breaks are observable using polyacrylamide gel electrophoresis (PAGE) if the DNA is end-labeled. This process is often referred to as Maxam−Gilbert G-lane chemistry (*26*, *28*). For sequencing proposes, the use of DMS is ideal because it alkylates all G's to a similar extent and provides a uniform G ladder.

The lack of sequence selectivity turns out not to be the case for all methylating agents. It was first noticed by Briscoe and Cotter that the yield of methylated guanines in MNU-treated DNA was sequence-dependent with poly(dG)•poly(dC) being methylated 2-fold more than poly(dG-dC), poly(dA-dC)•poly(dG-dT), and poly(dA-dG)•poly(dC-dT) (*29*). We showed that MNU, which reacts with DNA via a methanediazonium ion ($CH_3N_2^+$) intermediate (*30*), produces a clear sequence-dependent methylation pattern (Figures 3 and 4) (*31*). The regiospecificity approaches 10-fold between the most and least reactive G's within restriction fragment size DNA. To demonstrate that the specificity was due to an interaction of the $CH_3N_2^+$ with DNA, rather than some intermediate precursor, we substituted in place of MNU a number of compounds that generate $CH_3N_2^+$ and studied the reaction products. Diazomethane, acetoxymethylmethylnitrosamine, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine all gave identical 7-G methylation patterns and the same yield of 7-mG (Figure 4) (*32*). Therefore, the sequence selectivity is due in part to some characteristic of the $CH_3N_2^+$. The gel fragmentation pattern indicates that a G that is on the 3′-side of another G is generally the most reactive site for $CH_3N_2^+$, and the order of reactivity is 5′-G*G* > 5′-A*G* > 5′-C*G* > 5′-T*G*. The 3′- base has a smaller effect and follows the order of *G*G-3′ > *G*A-3′ > *G*T-3′ > *G*C-3′.

Virtually the same alkylation pattern seen with $CH_3N_2^+$ was also observed for a number of 2-chloroethylating compounds: nitrogen mustards (*33*), *N*-nitrosoureas (*34*, *35*), and triazenes (*36*). In the case of the nitrogen mustards, it was reported that attaching an N-substituted uracil or quinacrine group altered the specificity of the alkylation pattern, presumably due to weak equilibrium binding interactions of the heterocyclic ring of the mustard to selective DNA sequences prior to the alkylation event (*37*). There are more striking examples of changes in the methylation pattern when an *N*-methyl-*N*-nitrosourea functionality is linked to DNA equilibrium binders. Attaching the nitrosourea to a methidium intercalator causes the methylation pattern at N7-G to become uniform (*38*). In contrast, using a minor groove binding peptide to deliver the alkanediazonium afforded a very significant increase in the amount of N3-alkyladenine relative to free nitrosoureas (*39*, *40*).

There have been numerous efforts to explain and predict DNA alkylation patterns by nitrosoureas. The Pullmans' calculated the electrostatic potential at the different bases and the influence of flanking bases on the potential (*19*, *41*). Although their calculations provided the initial insight into the electrostatic environment in the grooves of ds-DNA, the approach was limited in being able to predict alkylation patterns. Klopman et al. attempted, with limited success, to relate the alkylation patterns to the effects of base stacking on reaction potentials (*42*). Another approach to the question was proposed by LeBreton who tried to relate the reactivity at different G's to

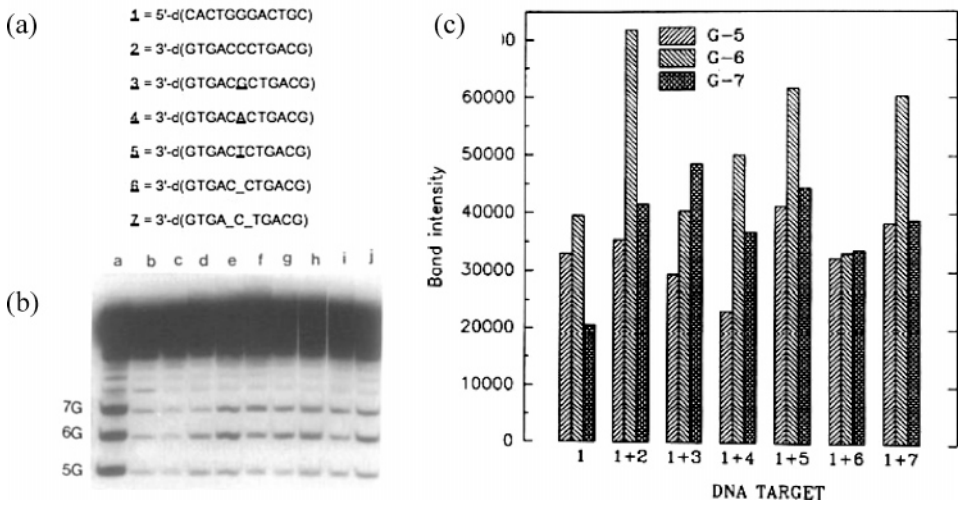

Figure 5. Methylation patterns of *N*-methyl-*N*-nitrosourea (MNU) in ss-DNA, ds-DNA, and in ds-DNA with mismatches and bulge sites (*47*). (a) Sequences of oligomers. (b) Gel pattern; lane a, G-lane; lane b, G + A; lane c, control; lane d, *1* + 500 μM MNU; and lane e, *1* + *2* + 500 μM MNU; lane f, *1* + *3* + 500 μM MNU; lane g, *1* + *4* + 500 μM MNU; lane h, *1* + *5* + 500 μM MNU; lane i, *1* + *6* + 500 μM MNU; lane j, *1* + *7* + 500 μM MNU. (c) Densitometry analysis of the gel in b.

an increase in π polarizability in the highest HOMO of G that reduces the transition state activation involving charge transfer (*43*−*45*). It was calculated that the ionization potential of G, which has the lowest ionization potential of the nucleobases, is decreased when it is in the interior of a hyper-reactive G_{3-4} stretch. The integration of all of these theoretical studies and other experimental work, including the data presented below, is consistent with an electrostatic association of the charged alkylating intermediate that is sequence-selective.

The alkylation studies described above were performed in vitro; therefore, an obvious question is whether the same adduct patterns would be observed in vivo because in the latter case, the DNA is associated with proteins, including those involved in the formation of nucleosomes. It was demonstrated using ligation-mediated PCR that both MNU and *N*-nitroso(acetoxymethyl)methylamine afford the same methylation pattern at N7-G in human cell lines as they do in solution (*46*). This result indicates that in the millions of cells exposed in vivo to an alkylating agent, protein occupancy varies at any particular DNA sequence, that is, in some cells, a DNA site may be bound by protein, and in others the DNA sequence is unbound. At low doses of alkylating agent, that is, low levels of DNA modification, the availability of unbound sequences in DNA is in large excess. This does not mean that methylation in a single cell and at a specific sequence will not be affected by the presence of a DNA binding protein. It simply means that within the environment of the nucleus of a cell, the factors responsible for the alkylation patterns are the same as those in naked DNA.

4. Effect of DNA Structure and Conformation on Alkylation Reaction

To determine if the sequence-dependent reaction of MNU stemmed from steric influences in the major groove, we studied the alkylation pattern of MNU in single-strand (ss) DNA. The pattern observed in ds-DNA is lost in ss-DNA, and most importantly, the alkylation yield was significantly reduced (Figure 5) (*47*). In contrast, DMS, which shows no sequence selectivity with either ss- or ds-DNA, reacts more efficiently with the former. The greater reactivity of ds-DNA relative to ss-DNA suggests that steric factors are not important determinants in the sequence selectivity for MNU. This indicates that the formation of the double helix from ss-DNA creates a sequence-dependent electronic environment to which some alkylating agents, that is, those intermediates with cationic character, are sensitive.

To test whether more subtle disruptions of the canonical Watson−Crick helix would affect the methylation pattern, we reacted MNU with duplex oligomers with and without mismatches at the central G of a G_3 target sequence. Consistent with the requirement for a canonical double helix, a G that is mispaired with G, T, or A becomes less reactive than a G paired with C (Figure 5) (*47*). Similarly, the creation of G's in a single or double bulge show a decrease in alkylation (Figure 5). In all cases, the DNA retains the B-conformation based upon the CD spectra, although the base stacking, measured using the negative band at 240 nm, is somewhat reduced in the noncanonical structures. The results show that a noncanonical structure significantly reduces the methylation at the G that is at a mismatch or bulge, whereas the effect at flanking G's is relatively small. To obtain further verification that steric factors were not in play in the sequence selectivity of MNU, DNA was treated with 2,12-dimethyl-3,7,11,17-tetraazobicyclo-[11.3.1]-heptadeca-1- [17],2,11,13,15 pentaene-Ni(II) in the presence of $KHSO_5$. This bulky agent is quite sensitive to steric accessibility and has been used to mark noncanonical DNA base pairing motifs (*48*). The data from these studies confirm that there is no correlation between the steric accessibility of the Ni(II) complex and the electrophilic reactivity of MNU. A final set of experiments that show the preference for a duplex is the MNU-methylation of DNA over a temperature range from 0 to 80 °C. As the temperature of the reaction is raised and the DNA starts to denature, the pattern disappears, and the alkylation intensity decreases.

It has also been reported that the reaction of calf thymus DNA, supercoiled plasmid DNA, and synthetic oligomers show similar alkylation patterns when treated in vitro with $CH_3N_2^+$ (*49*). Therefore, the topology of the DNA target does not affect reactivity, assuming that the DNA retains a B-conformation. There is one study on the methylation of putative Z- and H-DNA by $CH_3N_2^+$ that reports that the difference in methylation was minimal (*50*). However, in these studies, the Z- and H-conformations of oligomeric inserts in plasmid DNA were induced by topoisomerase I-catalyzed relaxation of the DNA in the presence of different concentrations of ethidium bromide.

Therefore, it is likely that a mixture of B- and Z- or H-conformations was present in the methylation reactions. Because only a fraction of the DNA is alkylated in the sequence studies, the more reactive conformation, possibly B-DNA, will be preferentially methylated. In the same studies, DMS showed no change in its reactions with putative H-DNA. Clearly, in H-DNA, the steric accessibility to major groove atoms should have been significantly impeded because the reduced accessibility to alkylating agents is used in footprinting experiments as proof of H-DNA structure (*51*). Therefore, the effect of conformation on the DNA methylation pattern by charged alkylating intermediates remains unresolved.

5. Effect of Salt on DNA Alkylation

DNA methylation reactions were carried out in the presence of various inorganic and organic salts and at various salt concentrations to determine the nature of electrostatic interactions between atoms in the major groove and the $CH_3N_2^+$ intermediate (*31*, *32*). The level of DNA methylation was inversely dependent on salt concentration and the binding affinity of the cation; however, the methylation pattern itself remained unperturbed (Figure 3). Other laboratories observed an inhibitory effect of distamycin A on adduct yields but attributed the inhibition to its minor groove binding properties (*52*). However, we showed that the effect was on both major and minor groove products and that the former inhibition was independent of sequence, whereas the latter occurred at distamycin A equilibrium binding sites (*53*). The degree of inhibition followed what would be predicted from the equilibrium binding constants of the cations (*32*). Accordingly, it requires approximately 10 mM $MgCl_2$ to inhibit N7-G methylation by MNU to the same extent as 200 mM NaCl (*32*). The addition of salt does not affect DNA methylation by DMS (Figure 3) (*31*).

These studies confirmed that there is an intrinsic sequence-dependent electrostatic field in the major groove, irrespective of the ionic strength of the buffer or the nature of the diffusible cations present. We have termed this the sequence-dependent electrostatic landscape (SDEL) of DNA (*54*). We propose that the SDEL is, to a large measure, responsible for the sequence-dependent alkylation of DNA by charged intermediates as well as the equilibrium binding of small cationic molecules to DNA.

6. Alkylation Patterns at Other Positions

Are sequence-selective events that occur at N7-G unique? Another DNA lesion generated by MNU is *O*^{6}-methylguanine (6-mG). Although it is formed at 10% of the level of 7-mG, its biological role in toxicity and mutagenicity is well-documented (*17*, *55*−*57*). Because 6-mG is relatively stable to chemical approaches to selectively cleave it off DNA and because it is formed along with the more labile 7-mG adduct, it is not possible to directly and unambiguously sequence 6-mG using standard chemical approaches. By isotopically labeling the individual G's in a short duplex, two groups have been able to determine the sequence selectivity for 6-mG formation (*58*, *59*). Both studies show that the patterns observed at 7-G and 6-G are similar. Thus, the same electrostatic factors are at work in determining the electrophilic reactivity of the two major groove G atoms that are just under 3 Å apart. The mechanistic factors responsible for the partitioning of the alkanediazonium ion between the reaction at N7-G versus that at *O*^{6}-G remain unclear, although recently, a model involving electrostatics has been suggested (*60*).

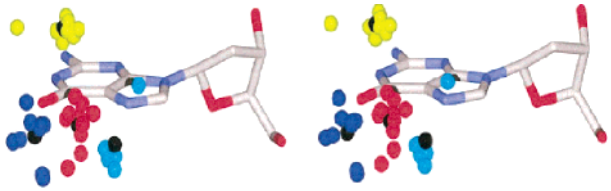

Figure 6. Relaxed stereoview of cation localization at consensus sites obtained from the superimposition of guanine residues in the protein database that are adjacent to monovalent cations (*54*). This composite shows that G's are high occupancy cation binding sites, even for monovalent cations: blue, near O^6-G; red, near both O^6- and N7-G; cyan, near N7-G; and yellow, near both bases in the dinucleotide step (generally O^6- and N7-G).

In contrast to the sequence dependency observed in the major groove, N3-methylation of A in the minor groove of restriction fragment length DNA is virtually uniform (*49*). Only in the presence of minor groove binding ligands does the alkylation pattern become nonuniform, and this coincides with the steric inhibition effect of the bound ligand at specific minor groove sequences.

7. Electrostatic Binding of Cations to DNA

The factors governing the sequence-selective methylation of DNA at N7-G and at O^6-G suggest that there is an electrostatic code associated with ds-DNA (the SDEL) that is sensed and covalently marked by $CH_3N_2^+$ and related alkylating agents. If so, then inorganic cations might be expected to preferentially associate with the same regions. Is there any evidence for this? The B-form helix is a polyanion with characteristic axial charge density. In the theory developed by Manning and others, this results in the condensation of diffusible cations in a region proximal to the DNA, with an effective neutralization of approximately 75% of the formal anionic charge (*61*−*63*). The theory predicts that this condensation effect is a function of the axial charge density and is constant over a broad range of bulk cation concentration. This type of electrostatic cation association is not predicted by the theory to exhibit any sequence dependency; the sequence does not change the axial charge density, as confirmed by high-resolution NMR and X-ray crystallography studies (*61*). To date, these cations associated with the phosphate backbone have been infrequently observed using either high-resolution structural method.

Monovalent cations are sporadically observed in high-resolution X-ray crystal structures of DNA, primarily in the major groove near G−C pairs and in the minor groove near A−T pairs (Figure 6) (*54*, *64*). Cations in the major groove cluster within 3.5 Å of the N7- and O^6-positions of G, and in some cases, they appear as bifurcated bridges between the two electronegative atoms on the same G or between the atoms on neighboring G's. The use of Tl^+ as a K^+ substitute because of its anomalous scattering has facilitated the accurate mapping of monovalent cation locations (*65*). Monovalent Na^+, K^+, Rb^+, Cs^+, and NH_4^+ ions and H_2O molecules, and even polyamines and divalent cations, compete for similar sites on DNA or RNA (*65*−*67*). Various species bind with partial and mixed occupancies in X-ray structures. Na^+ and NH_4^+ scatter X-rays with nearly the same power as that of water or partially occupied K^+. Each of these species has irregular and variable coordination. Therefore, the solvent/ion environment can be difficult to fit unambiguously during structure refinement. A well-developed K^+ substitute with a distinctive X-ray scattering fingerprint that obviates the interpretation of subtle differences in coordination geometry and scattering power is provided by Tl^+. Tl^+ and K^+

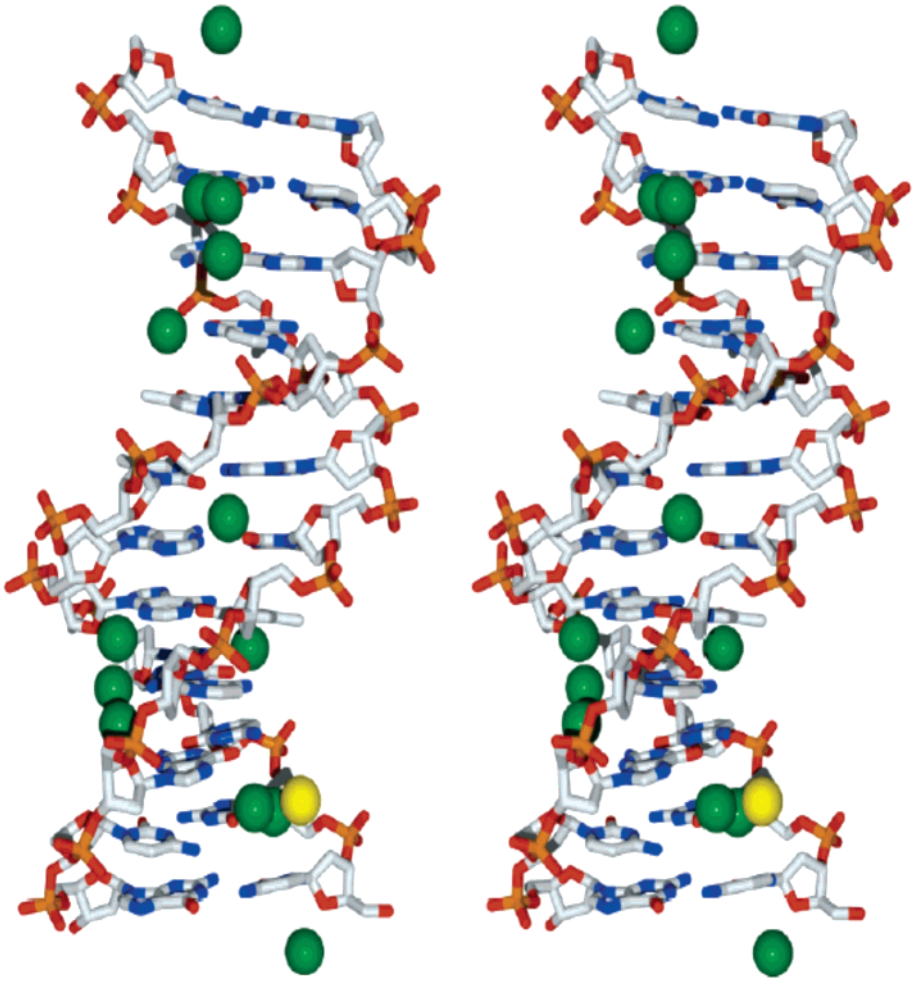

Figure 7. Relaxed stereoview of 5′-d(CGCGAATTCGCG)$_2$ (DDD) and associated Tl^+ ions based upon 1.2 Å resolution X-ray data (*65*). Tl^+ ions are depicted by green spheres, and the sole Mg^{2+} ion is depicted by a yellow sphere. DNA is shown in stick representation with standard CPK color coding of atoms. H_2O molecules have been omitted for clarity. For reference, the minor groove is in the vertical center of the DNA molecule.

have similar ionic radii (K^+ = 1.33 Å; Tl^+ = 1.49 Å) (*68*) and enthalpies of hydration (K^+ = −77 kcal mol^{-1}; Tl^+ = −78 kcal mol-1) (*69*). Tl^+ can substitute for K^+ in the catalytic mechanisms of sodium-potassium pumps (*70*), fructose-1−6-bisphosphatase (*71*), and pyruvate kinase (*72*). Tl^+ has been shown by NMR to stabilize guanine tetraplexes in a manner analogous to that of K^+ and NH_4^+ (*73*). We have used Tl^+ as a marker for sites of K^+ localization adjacent to B-DNA (*65*) and DNA−drug complexes (*54*). Doudna and co-workers used Tl^+ as a marker for sites of K^+ localization tetrahymena ribozyme P4−P6 domain (*74*). Tl^+ was used by Caspar and co-workers to determine counterion positions adjacent to insulin (*75*, *76*) and by Gill and Eisenberg to determine the location of NH_4^+ ions in the binding pocket of glutamine synthetase (*77*).

The self-complementary Drew−Dickerson dodecamer (DDD) (Figure 9) has been crystallized in the presence of Tl^+ (*65*). The locations of monovalent and divalent cations are depicted in Figure 7. In addition to the location, the X-ray structure also provides information on the occupancy of cations at the different sites. The highest occupancy is calculated to be at the C^9−G^4 pair. One significant caveat of these crystallographic studies is that the DNA sequences studied using high-resolution crystallography are quite limited. Structures of the sequences that are hot spots for alkylation (and mutagenesis) have not been determined. Despite this limitation, it is reassuring that the relative occupancies of inorganic cations in the major groove of DDD correlates well with the methylation intensities (Figure 8). The methylation data show that the yield of 7-mG at G^4 is 25% higher than at G^2 and G^{10}, and G^4 is associated with the highest Tl^+ occupancy derived from X-ray crystallography.

In a series of line-broadening NMR studies, Braunlin's group (*78*−*80*) showed that di and trivalent ions, such as Mg^{2+}, Ca^{2+}, and Co^{3+}, associate with DNA with distinct sequence preferences related to G/C content. As with the crystallographic results described above, these NMR results are not predicted by the counterion condensation theory of Manning (*61*) or by the cylindrical Poisson−Boltzmann cell model (*81*). Braunlin proposed that neighboring G's might provide favorable divalent cation binding sites via interaction with the major groove N7 and O^6 atoms (*79*). These same G/C sequences have also been associated with cation-mediated bending in crystal structures (*66*, *70*, *82*−*84*). It is important to point out that using the same NMR line-broadening technique and $^{23}Na^+$, the Braunlin group found no evidence for a G/C association preference (*85*). As mentioned above, monovalent cations are also seldom observed in high-resolution crystal structures of DNA, but when they are observed, they are at many of the same regions that divalent cations bind, that is, at G/C sequences. This is not to say that hydrated divalent cations and weakly hydrated divalent and monovalent cations uniformly have the same DNA association characteristics.

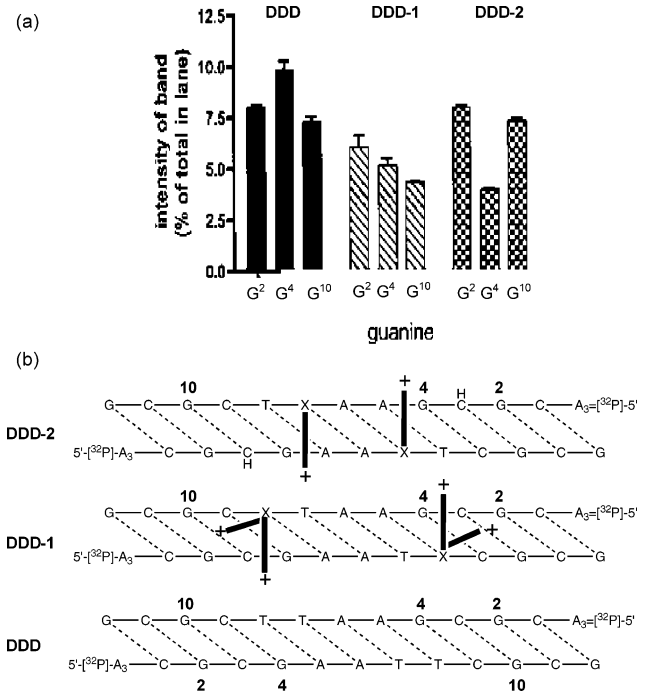

Figure 8. (a) Methylation pattern observed in unmodified DDD and 3-aminopropyl-dU-modified DDD-1 and DDD-2. (b) Depiction of the position of side chains based upon the methylation inhibition pattern.

DDD: 5'-d(CGCGAATTCGCG)
DDD-1: 5'-d(CGCGAATXCGCG)
DDD-2: 5'-d(CGCGAAXTCGCG)
DDD-3: 5'-d(CGCGAAXXCGCG)

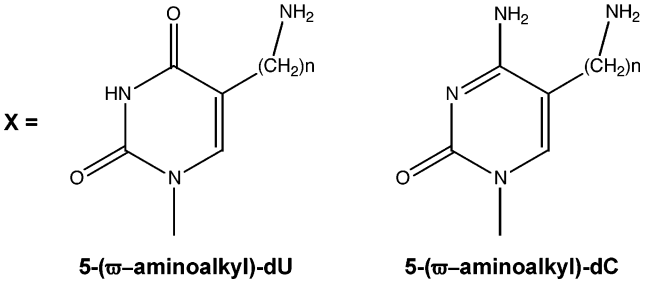

Figure 9. Structures of dodecamer sequences and modified bases used in study ($n = 3$ or 6).

8. Probing and Manipulating the Electrostatic Potential of DNA

The SDEL appears to modulate or even drive the major groove alkylation pattern as well as influence cation association. Therefore, experiments were designed to manipulate the electrostatic environment to determine if, and how, DNA alkylation would be affected. This was done by covalently tethering cations at specific sites using an ω-aminoalkyl side chain at the 5-position of a pyrimidine base (Figure 9).

The 5-(ω-aminoalkyl)pyrimidines, which can conveniently be introduced into DNA oligomers at specific positions using standard phosphoramidite chemistry, have been previously prepared and used to provide a primary amino group for postcolumn modification with a number of functionalities, including anisotropy probes (*86*). Originally, it was assumed that in ds-DNA, the terminal ammonium ion in ω-aminoalkylpyrimidines would form a salt bridge with the 5′-nonbridging phosphate oxygen (*87*−*89*). However, this conformation appeared to be unlikely on the basis of molecular simulations and grid search computational approaches that predicted that the alkyl tethered ammonium ion would associate with major groove N7- and O-G sites that were on the 3′-side of the modification (*90*). Replacement of the ω-amino functionality with an ω-hydroxyl in the simulations reduced the interaction between the atoms on G in the major groove and the side chain, but the side chain still retained the 3′-orientation. The reaction of MNU with oligomers modified with the 5-(ω-aminoalkyl)-2′-deoxypyrimidines revealed that there was selective inhibition of methylation directed toward the 3′-direction, which is what was predicted from molecular modeling (Figure 8) (*91*). The site of inhibition of 7-mG formation could be controlled by shortening or lengthening the aliphatic tether between the pyrimidine and the ammonium ion (*90*). Significantly, the cationic side chain has virtually no effect on DNA methylation by DMS, which is consistent with electrostatic repulsion of the tethered cation with the attacking $CH_3N_2^+$ but not with DMS. It is not clear whether the tethered ammonium ion prevents alkylation by electrostatically repulsing the $CH_3N_2^+$ and/or whether it tightly binds to the N7- and O^6-positions on the G's and sterically blocks the covalent attack. Because DMS is not affected by the presence of the side chain, the former electrostatic repulsion scenario appears more likely because methylation by DMS would also be sensitive to the presence of the tethered cation stably interacting with N7-G.

9. Localization of Change in the Major Groove Charge and DNA Structure

In analyzing the alkylation data, it was observed that inhibition of methylation occurs at sites that are too remote from the cationic adduct to be affected by steric or short-range electrostatic interactions in canonical B-DNA (*90*, *91*). The 3-aminopropyl side chain can physically extend approximately 4 Å through space, but inhibition is seen two to three bases pairs away, which is equivalent to >8 Å in B-DNA. The 6-aminohexyl side chain, with its six-atom bridge, can extend ∼8 Å above the plane of the pyrimidine ring, yet it influences methylation three base pairs away, or >11 Å. The unanticipated electrostatic reach of the tethered cations suggested that they deform DNA from the classical B-conformation. There is precedence for the ability of cations to induce changes in DNA structure. The same 5-(ω-aminoalkyl)-2′-deoxypyrimidines, when appropriately phased so that they are on the same face of the double helix with an inherently bent A-tract, cause increased aberrant gel mobility (*88*, *89*). This aberrant mobility is generally interpreted to indicate that the DNA has a static bend or anisotropic flexibility (*92*). The bending was reduced when the 5-(ω-aminoalkyl)-2′-deoxypyrimidines were orthogonal to the A-tract, and when trans, the bending disappeared. Increasing the salt concentration also reduces bending, which implies an electrostatic role in the bending (*88*, *89*). More recently, the incorporation of the same 3-aminopropyl-dU residues were shown to shorten the 5′−3′ length of DNA using fluorescence resonance energy transfer (*93*). In these studies, two adjacent cationic side chains were required to observe measurable bending, and it was proposed that the cations had a cooperative effect. As mentioned above, divalent cations, which preferentially bind at G-rich sequences (*77*−*80*), appear to bend DNA at these sequences (*82*).

There are additional cases where the introduction of charge in the major groove causes dramatic bending. The interstrand cross-links formed by nitrogen mustards, for example, mechlorethamine, were predicted to form between the N7-positions of G's in 5′-GC sequences because the G's are appropriately spaced

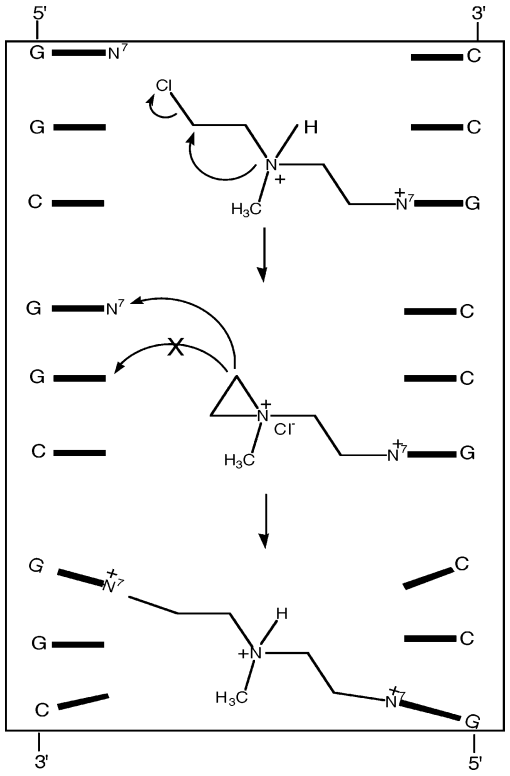

Figure 10. Interstrand cross-linking by mechlorethamine. The monofunctional lesion (top), which has a cationic purine and a cationic side chain, distorts the DNA; therefore, the 5′-GNC cross-link forms in favor of the predicted 5′-GC lesion (middle). The distance of the 5′-GNC cross-link is ~1.5 Å longer than the covalent bridge between the strands; therefore, the DNA must be distorted (bottom).

to accommodate the bridging -$(CH_2)_2$-N(CH_3)-$(CH_2)_2$- linkage without distorting the B-DNA structure (*94*). However, it was independently shown by the Loechler and Hopkins labs that the major site for interstrand cross-linking was at N7-G in 5′-*G*NC sequences despite the fact that the cross-linked DNA is highly distorted (*95*−*98*). Distortion is required because the covalent linkage between the two strands is at least 1.4 Å too short to accommodate a classical B-DNA conformation (Figure 10). A priori, there are two explanations for the unanticipated cross-link sequence specificity: (i) DNA normally adopts an array of nonclassical structures and the 5′-GNC cross-link is the kinetically favored product; or (ii) the initial monofunctional mustard adduct stabilizes a conformational perturbation in B-DNA that allows the 5′-GNC cross-link to efficiently form. The first explanation is not in accord with the time-averaged structures on the μs time scale obtained by NMR, nor are the static crystal structures, which also do not reveal the presence of highly distorted structures (*64*). In addition, other techniques such as fluorescence resonance energy transfer (*99*) and electron paramagnetic resonance (*100*) also do not indicate the degree of conformational diversity in DNA that is required for the 5′-GNC cross-link.

The initial reaction of nitrogen mustards with DNA results in a monofunctional N7-G adduct that has both a cationic purine ring and an ionized amino group on the side chain appendage (Figure 10). To dissect the role of the cationic purine on the sequence specificity of DNA interstrand cross-linking, we studied a series of α,ω-alkanediol dimethylsulfonate esters, CH_3-SO_2O-$(CH_2)_n$-OSO_2CH_3 (n = 4−6 or 8) (Figure 11) (*101*). These compounds, some of which have been clinically used to treat acute myeloid leukemia (*102*), cross-link DNA via monofunctional cationic N7-alkylG adducts, which do not have

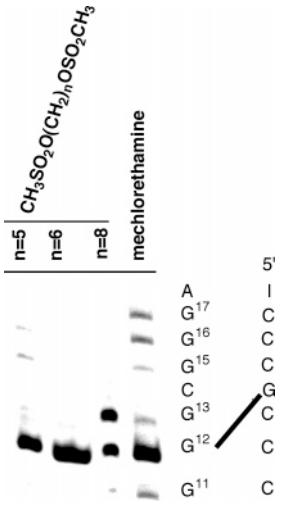

Figure 11. Cross-linking specificity of bis-sulfonates in 5′-[^{32}P]-A_2C_2-$TATAT_2G_3CG_3AT_2A_2$ (*101*). The gel isolated cross-link products are treated with piperidine to generate strand breaks at N7-alkyl guanine lesions. The results indicate the preference for the 5′-GNC cross-link (G-12 band) for n = 5 and 6 but an equal amount of cross-link at 5′-GNC and 5′-GC for n = 8.

cationic side chains (*103*). The sequences shown in Figure 11 were treated with one of the sulfonate esters or mechlorethamine. The cross-linked DNA and noncross-linked DNA were separated on nondenaturing gels. Quantification of the bands gives the relative cross-linking efficiency of sulfonate esters with 6-, 5-, 8-, and 4-carbon linkers as 22:6:1:0, respectively (*101*). To determine if the preferred cross-link site reflects the sequence-selective formation of the monofunctional precursor, the locations of the monofunctional lesions were also quantified. The results indicate that the very weak sequence selectivity for monofunctional adduction by the sulfonate esters and the strong specificity for cross-linking do not overlap. Because the linker is too short to span the modification sites of the reactant B-form DNA, the DNA must be distorted in an intermediate state, prior to the closure of the second linkage, so that the two atoms involved in bond formation are close enough to efficiently form a transition state on the cross-linking pathway. The presence of a cationic nucleobase (i.e., N7-alkyl-dG) is sufficient to induce distortion in the DNA so that the GNC cross-link is efficiently formed. This conclusion is consistent with the similar sequence specificity of diepoxybutane cross-linking, which also occurs at N7-G's in 5′-GNC (*104*). Therefore, the deformation of the cross-link is independent of the alkylating agent; an initial charged N7-G monofunctional intermediate is sufficient.

10. Structural Studies on DNA with Tethered Cationic Sidechains

To understand the affects of tethered cations, such as in 5-(ω-aminoalkyl)-2′-deoxypyrimidine on DNA methylation and conformation, structural studies using both NMR and X-ray crystallography were initiated. We chose the DDD sequence mentioned above because even when chemically modified, it frequently affords crystals that provide high resolution information. The first structure analyzed was 5′-d(CGCGAATXCGCG) (DDD-1), where X is 5-(3-aminopropyl)dU. NMR data indicate that the amino moiety is oriented in the 3′-direction from the site of modification (*105*), which is the same preference seen in the MNU methylation and molecular modeling studies (*90*, *91*). Structures emergent from molecular dynamics calculations in which the amino group was constrained to be proximate to the O^6-position at G^{10}, on the floor of the major groove, were

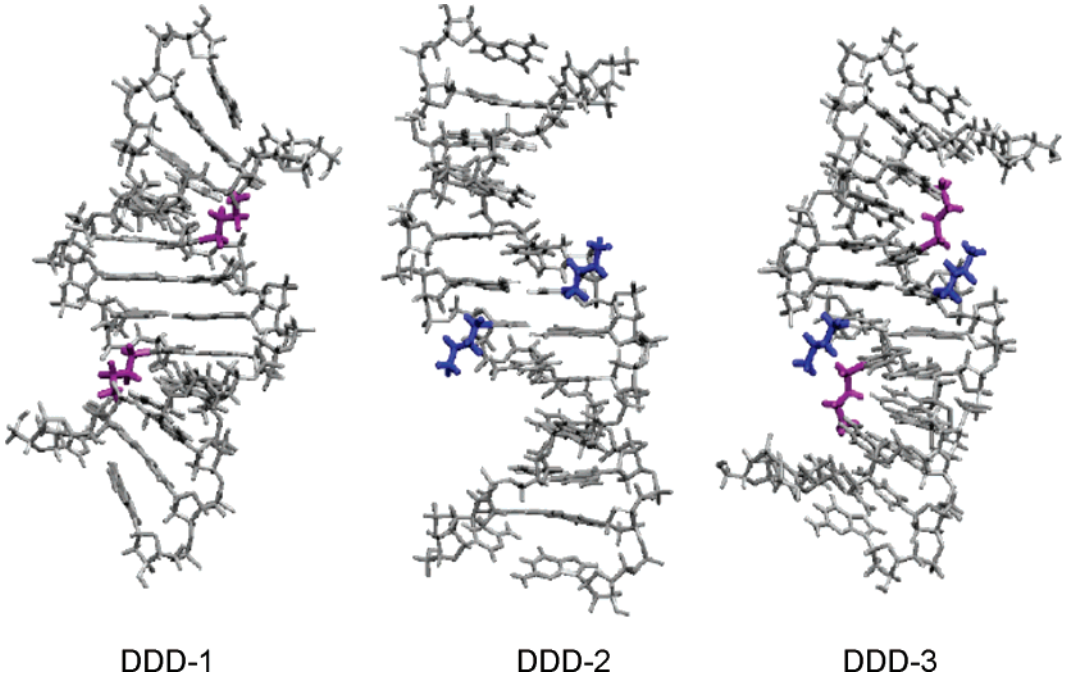

Figure 12. NMR derived structures of DDD-1 (*105*), DDD-2, and DDD-3. The purple side chains are attached to position X-8, and the blue side chains are attached to X-7 (Figure 9).

consistent with experimental NOE's and indicated the possibility of axial bending (Figure 12). The interpretation of the data that emerged from electrostatic footprinting (*90*, *91*) and NMR (*105*) studies posited that as a consequence of favorable electrostatic interactions, the tethered amine occupied the conformational space in the vicinity of O^6 and N7 of G^{10}, inducing DNA bending, and enabling the release of a diffusible cation from a high-affinity major groove cation binding site associated with the CGCG tracts of the DDD (*65*). Indeed, as mentioned above, the preferential binding of monovalent cations within the major groove in CGCG has been substantiated by crystallographic analysis in the presence of Tl^+ (Figure 7) (*65*).

The model of the tethered cation interacting with a site near G^{10} is also supported by molecular dynamics simulations of solvated oligodeoxynucleotides with counterions (*106*−*109*). Auffinger and Westhof (*110*) suggested preferential cation binding near the floor of the major groove adjacent to the edges of G−C base pairs close to the O^6- and N7-G. These cations exhibited significant residence life-times (ca. 500 ps). Additional NMR studies on DDD-1 involving the measurement of residual H3′-^{31}P dipolar couplings were performed, and the data confirm the conformation preference of the 3-aminopropyl side chain.

11. Effect of Cation Localization in the Major Groove on DNA Stability and Conformation

To fully understand how the sequence-specific introduction of cationic charge in the major groove affects local DNA structure, including the organization of cations and water, a detailed thermodynamic characterization of DNA containing the tethered cations was initiated. It was equally important to correlate the thermodynamic effects of the incorporation of charged side chains with the structural characteristics of similar DNA complexes. For this reason, we used a combination of optical and calorimetry techniques to investigate the helix−coil transition of DDD and DDD-1 and to characterize the counterion and water releases accompanying the unfolding of these duplexes (*111*). The comparison of the resulting thermodynamic profiles yields the specific energetic, ion, and hydration

Table 1. Thermodynamic Profiles for the Formation of DDD, DDD-1, DDD-2, and DDD-3 at 20 °C (*111*)[a]

duplex	T_M (°C)	ΔH_{cal} (kcal/mol)	$\Delta G°_{cal}$ (kcal/mol)	$T\Delta S_{cal}$ (kcal/mol)	Δn_{Na^+} (per/mol)	Δn_W (per/mol)
			Duplex → Single Strands			
DDD	33.3	−116	−6.9	−109	−2.3	−38
DDD-1	29.8	−68	−3.3	−65	−1.5	−7
DDD-2	37.3	−53	−3.7	−49	−1.7	−15
DDD-3	25.3	−50	−1.8	−49	−1.0	
			Hairpin → Single Strands			
DDD	62.8	−37	−5.3	−32	−0.2	−9
DDD-1	64.5	−27	−4.0	−23	−0.2	−6

[a]All parameters are measured from DSC and UV melting curves in 10 mM sodium phosphate buffer at pH 7.0. The experimental uncertainties are as follows: T_M (±0.5 °C), ΔH_{cal} (±3%), $\Delta G°_{cal}$ (±5%), $T\Delta S_{cal}$ (±3%), Δn_{Na^+} (±5%), and Δn_W (±8%).

contributions for placing a cationic chain at specific positions in the major groove of the DDD. A comparison of the melting characteristics of DDD and DDD-1 at 260 (unstacking of A−T and G−C pairs) and 275 nm (most sensitive to G−C unstacking) show that the denaturation behavior corresponds to sequential duplex → hairpin, and hairpin → random coil transitions. This is in agreement with earlier unfolding measurements of DDD (*112*). Placing a 3-aminopropyl-dU residue at X^8 drops the T_M by ∼3 °C relative to that of DDD. The thermodynamic parameters (Table 1) provide additional insights into the effect that the tethered cation has on the structure. There is an unfavorable $\Delta\Delta G°$ of 3.6 kcal/mol for DDD-1 relative to that of DDD resulting from a large compensation of an unfavorable $\Delta\Delta H_{cal}$ of 48 kcal/mol with a favorable $\Delta(T\Delta S_{cal})$ term of 44 kcal/mol. The differential enthalpy term indicates differences in base stacking and hydration between the two duplexes, whereas the differential entropy term is consistent with both a net counterion release of 0.8 mol Na^+/mol duplex and a release of 31 mol water/mol duplex. The overall effects are consistent with the tethered amino group directly or indirectly neutralizing charge, perhaps causing some increase in the exposure of aromatic nucleobases to the solvent, as would be anticipated if the DNA was bent. The indirect charge neutralization could

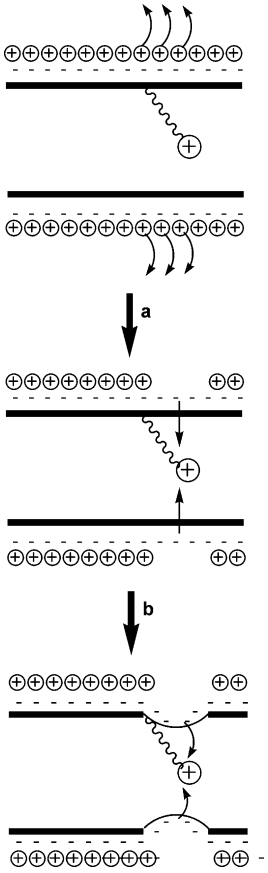

Figure 13. Proposed pathway to explain the bending induced by major groove localized cations. (a) Repulsion between the tethered NH_3^+ ion and diffusible cations that screen the nonbridging phosphate. (b) Collapse of the partially unscreened backbone onto the tethered cation (based upon ref *113*).

occur by the electrostatic mechanism proposed by Rouzina and Bloomfield (*113*). In this mechanism, the local presence of the cationic aminopropyl chain (rather than divalent cations used in the calculations of Rouzina and Bloomfield) located in the major groove of DNA regiospecifically repels phosphate screening counterions, and the unscreened phosphates collapse toward the propyl tethered ammonium ion (Figure 13). This scenario is consistent with our thermodynamic results and nicely explains how the incorporation of ω-aminoalkyl side chains can induce DNA bending.

If the aminopropyl-induced bending of the DNA, to which is attributed the slower gel mobility (*88*, *89*) and methylation inhibition pattern (*90*, *91*), is indeed driven by the tethered ammonium ion moving into a high-affinity binding site in the major groove, then altering the position of the modified base in the dodecamer should modulate the effect. Specifically, relocating the modification from position X^8 to position X^7 (i.e., 5′-CGCGAAX^{7}TCGCG, DDD-2) should prevent the tethered amine from reaching and displacing a bound cation in the major groove adjacent to N7 and O^6 at G^{10} because of the increased energetic penalty for the distortion of the DNA required for the 3-carbon tether to reach as far G^{10}. It should be noted that this additional one base pair offset is roughly equivalent to a $(CH_2)_3$ extension of the aliphatic tether between the base and the amine.

We synthesized DDD-2 and DDD-3 with 5-(3-aminopropyl)-dU modifications at X^7 and at both X^7 and X^8, respectively. In contrast to what was observed in the NMR spectra of DDD-1, when located at X^8, the cationic moiety in DDD-2 is not positioned toward the floor of the major groove (Figure 12). Furthermore, restrained molecular dynamics calculations yield

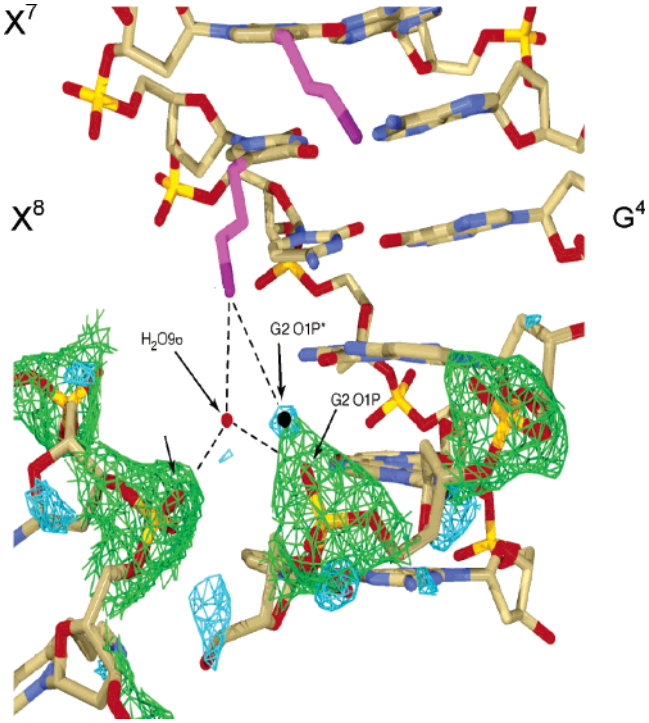

Figure 14. Close-up view of the contact between one of the amino propyl groups (in purple) attached to position X^8 and the backbone on the same DDD-3 molecule (Figure 9) with a symmetry related molecule (lower left). This side chain is near the floor of the major groove, whereas the other three point out into solution (*114*). The aminopropyl side chain attached to X^7 (upper center) can be seen pointing out from the major groove into the solvent near G^4.

ensembles of structures suggesting that DDD-2 is not bent. In DDD-3, the moiety located at X^8 behaves in a manner similar to that of the X^8 side chain in DDD-1, whereas the aminopropyl moiety located at X^7 behaves in a manner similar to that of the X^7 side chain in DDD-2. In DDD-3, bending is proposed on the basis of the NMR data, which is in agreement with that observed for DDD-1 (Figure 12). Overall, the comparison of modified DDD-1, -2, and -3 indicates that only the cation attached via X^8 induces bending, whereas placing the cationic moiety at X^7 does not.

While the NMR and thermodynamic experiments were being performed, we were able to get diffracting crystals for DDD-3. The results of this work are generally consistent with the NMR picture in that one of the tethered ammonium ions at X^8 points down into the groove and approaches G^{10} (*114*). The X^8 on the complementary strand, which should be identical, points out into the solution. In contrast, both of the ammonium ions tethered to X^7 point out into the solvent (Figure 14). The reason that the structure displays asymmetry is unclear. It may result from forces exerted by the crystal lattice or from an equilibrium between the two predominant DNA conformations: bent with side chain partially occupying the major groove cation site or linear with the side chain pointing out into the solvent. Using a combination of modifications that precisely positions cation charge in the major groove, for example, 7-aminomethyl-7-deazaguanine, or deletes the potential cation binding site near N7 and O^6-G, for example, 7-deazaguanine, we are trying to resolve this question.

Using the MNU ($CH_3N_2^+$)-based electrostatic footprinting approach (*91*), we characterized the position of the tethered ammonium ion in DDD-2 (Figure 8). In comparison to DDD, the location of the modification at X^8 (DDD-1) results in a 45% decrease in methylation at G^4 and G^{10} with no significant

difference at G^2. The data are interpreted to mean that the aminopropyl side chain in DDD-1 is conformationally flexible and moves between G^{10} and G^4. Attaching the side chain to X^7 (DDD-2) induced a >50% decrease at G^4 with no effect at the other G's. This is what would be expected if the side chain in DDD-2 predominantly pointed out into solution, which would situate it in the vicinity of G^4. Therefore, the electrostatic footprinting results are consistent with a dynamic side chain in DDD-1, where the cation moves down into the groove near G^2 or extends out into solution near G^4. In DDD-2, only the latter occurs.

12. Effect of Sequence and Cation Position on DNA Stability

Comparative thermodynamic analysis of DDD, DDD-1, DDD-2, and DDD-3 provided insight into how the movement of the aminopropyl side chains was affecting the stability and organization of cations and water in the duplexes. When the aminopropyl group is located at X^8 (DDD-1), the T_M drops by 3 °C relative to that of DDD (Table 1) (*111*), whereas placing it at X^7 (DDD-2) increases the T_M by 4 °C relative to that of DDD. Therefore, moving the 3-aminopropyl-dU−A pair by one position (~3.5 Å) causes a >7 °C change in T_M. The introduction of two modified residues on each strand (DDD-3) causes the T_M to decrease by ~8 and 11.5 °C relative to those of DDD and DDD-2, respectively. DDD-2 yields a free energy destabilization similar to that of DDD-1 ($\Delta\Delta G° = 3.2$ kcal/mol), but there is a larger enthalpy−entropy compensation ($\Delta\Delta H_{cal} = 63$ kcal/mol and $\Delta(T\Delta S_{cal}) = 60$) and a lower ion ($\Delta\Delta n_{Na+} = 0.6$) and H_2O ($\Delta\Delta n_W = 23$) release. In DDD-3, where a total of four cationic side chains are present, the differential release of counterions is the only parameter that is additive (1.3 mol Na^+/mol duplex vs 1.4 mol Na+/mol duplex); all others parameters have small changes relative to those of DDD-2. The thermodynamic data demonstrate that the location of the cationic side chain has a pronounced impact on how it alters DNA stability and cation and water organization. The thermodynamic observations are consistent with the structural differences seen using NMR and crystallography.

In sequences other than DDD, the thermodynamic effect of the aminopropyl side chain has also been unpredictable: in some cases, stabilizing and in others destabilizing. For example, in the noncomplementary duplex, 5′-d(CGTAGXCGTGC)−3′-d(GCACGACTACG), the aminopropyl side chain stabilizes the duplex by 6 °C (*115*).

13. Significance

The SDEL provides a predictive and unifying model that reconciles and explains structural, reactivity, and thermodynamic data. We believe that we have made a compelling case for the importance of the SDEL in the binding and bonding of cationic molecules (and reactive intermediates) with DNA. In turn, the intrinsic sequence-dependent reactivity toward electrophiles, in part, determines the mutation patterns with molecules that react via charged intermediates. Moreover, the structure of the nucleobases and the base pairing schemes in DNA have remained invariant over billions of years of evolution, but the primary sequence and the resulting secondary and tertiary structures have co-evolved with the proteins that are involved in DNA metabolism (*116*). Whether the creation of sequence-dependent major (and minor) groove cation binding sites is part of the co-evolution process remains to be seen. Clearly, basic amino acid side chains that are introduced into DNA as a result of DNA−protein equilibrium binding would be expected to localize in these high cation occupancy sites because of the favorable enthalpic electrostatic interactions and the entropic release of localized cations. In addition, the formation or localization of cationic charge in the grooves of the double helix, due to covalent bonding or equilibrium binding by small molecules and proteins, can affect DNA topology. The latter is mechanistically linked to how some DNA binding proteins coordinate the transcriptional regulation of genes.

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