Interaction of DNA methyltransferase with aminofluorene and N-acetylamino fluorene modified poly(dC-dG)

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ABSTRACT

Poly(dC-dG) was reacted in vitro to yield templates containing similar amounts of aminofluorene (AF) or acetylamino fluorene (AAF) adducts. These modified poly(dC-dG) templates were tested in an in vitro DNA methylation system utilizing 1500-fold purified rat liver methyltransferase (DMase) to compare and quantitate the effects of these adducts on the kinetics of methylation and the interaction of DMase with such templates. Enzymatic methylation is severely impaired by arylamine adducts, with bound AF inhibiting more than AAF (relative V_{max} 0.24 for AAF-poly(dC-dG) and 0.066 for AF-poly(dC-dG)). The apparent k_m for the reaction is not appreciably altered by AAF modification: 10 μ M for dCdG dinucleotide units, but it is threefold lower (3 μ M) for AF-poly(dC-dG). In competition experiments it was demonstrated that a translocational block is imposed by the adducts. From differential salt inhibition assays and preincubation assays, no change in the ionic binding to the altered templates could be detected, which suggests that the enzyme interacts very strongly through hydrophobic interactions with the fluorene ring. Evidence that the fluorene ring is exposed is supported by circular dichroism spectra of the templates under the conditions of the assay, which indicated that the AF adducts do not appreciably change the normal B conformation of the template, while the template with 9.5% modification by AAF adducts adopted a Z form. These results suggest that the inhibitory effects of AAF and, in particular, AF upon DMase-catalyzed methylation reactions are not dependent upon helix conformation. Instead, they appear to depend upon DMase recognition of an altered dG base configuration, which is responsible for altered binding and methylation kinetics.

INTRODUCTION:

The covalent modification of DNA by reactive electrophilic metabolites of arylamine compounds, which are known to induce various structural and/or conformational changes in the helix, is generally considered to be the key step for initiating the carcinogenic process (1,2). In vivo microsomal oxidation to N-hydroxyl derivatives of the carcinogens AF and AAF is believed to be the first step for activation into the procarcinogens N-OH-AAF and N-OH-AF, respectively (1,3). Evidence suggests that N-OH-AAF (which is much less reactive with cellular macromolecules than N-OH-AF) is converted to a powerful mutagen either by a transacylase (4), sulfotransferase (5), carboxyesterase

(6), or deacetylase (7,8), the latter yielding N-OH-AF. In support of the latter pathway, in AAF or N-OH-AAF carcinogenesis 80%-90% of the fluorene bound to DNA is unacetylated (9). When a nuclear metabolizing system is utilized in vitro, this is reduced to 27% (10), but with a microsomal system as much as 95% of the fluorene bound to DNA is unacetylated (11). Two adducts, N-(deoxyguanosin-8-yl)-AAF and 3-(deoxyguanosin-N2-yl)-AAF, have been identified in vivo (19). The C(8) derivative accounts for over 80% of the adducts, while the exocyclic N(2) accounts for the remainder. Using the synthetic N-acetoxy-N-acetylaminofluorene (N-AcO-AAF) reactive derivative of AAF, the same proportions and types of adducts can be formed in vitro, while no adducts to dC could be detected (9,11). Conversely, N-OH-AF is reactive in vitro and yields exclusively the C(8) derivative, which is structurally identical to the alkali deacetylated N-(deoxyguanosin-8-yl)-AAF (13). Physicochemical studies on these two forms of C(8) adducts indicate that the acetylated form disrupts the normal anti sugar conformation to syn, which rotates the fluorene ring into an intercalating position (base displacement model [14]), while the normal B-DNA conformation is conserved. In the unacetylated form, the fluorene ring is somewhat buried from spectroscopic evidence that suggests that it interacts with the bases. However, the sugar does not adopt a syn conformation and with increasing AF modification a B $\rightarrow$ C transition in helix conformation is observed (15).

The polynucleotide poly(dC-dG) can, under various condition of high salt, organic solvents, or divalent cations, adopt a left-handed helical Z form in which the dG sugars are forced into a syn conformation while the dC sugars retain their anti conformation (16). By covalent binding AAF to dG residues a B $\rightarrow$ Z conformational shift is facilitated and the Z form stabilized (17,18). The effect of binding AF to dG in this polymer is to facilitate slightly the conversion to the Z form, but only at much higher salt or ethanol concentrations than with AAF (17). With a high degree of AAF modification (20%), poly(dC-dG) in 50 mM NaCl adopts the Z conformation, while with lesser degrees of modification the polynucleotide is partially in Z (19). However, AF modification under these conditions gives no indication of even partial Z conformation (17). Behe & Felsenfeld demonstrated that the methylation of dC to m⁵dC had the effect of facilitating the conversion of poly(dm⁵C-dG) to the Z form, which is sufficiently stabilized to exist in Z form under physiological conditions of salt and temperature (20).

In eukaryotic genomes a small proportion of dC are methylated enzymatically to form the modified base m⁵C, principally in sequences consisting of

5'dCpdG. Several lines of evidence have implicated the methylation of DNA with the regulation of differentiation, gene activity, and X chromosome inactivation (see reviews 21,22). Alterations in the characteristic genomic pattern of DNA methylation of differentiated cells has been proposed as a possible mechanism for the induction of neoplasia (23). We and others have provided evidence that for a large number of chemical carcinogens this correlation holds (24-27). During AAF carcinogenesis in rats or mice the DNA methylation levels are affected, resulting in hypomethylated tumor cells (26,27) and similarly for cells treated with N-AcO-AAF (28). Salas et al (29) and recently Pfohl-Leszkowicz et al. (30) have investigated the mechanism for the inhibition of DNA methylation by AAF modified DNA. In this study we address the same problem with respect to the possibility that the effect of AAF or AF adducts may be to induce a local Z conformation in the DNA, which would impair methylation. Secondly, we wish to compare the effect of AF with that of AAF, since the most common form of the adduct is unacetylated in DNA. Further, a highly purified preparation of DNA methyltransferase was used.

We investigated the interaction of 1500-fold purified DNA methyltransferase from rat liver with poly(dC-dG) that was reacted in vitro to form either AF or AAF adducts. This enzyme, which functions as an active dimer with molecular weight of 280 kd, appears to be the only major DNA methyltransferase activity in rat liver; and has a preference for methylating double-over single-stranded DNA and hemimethylated over nonmethylated DNA thus qualifying for the role of "maintenance methylase." The purification, physical, and catalytic properties are described in detail elsewhere (31). Our results indicate that it interacts with an altered affinity on AF- as well as AAF-modified poly(dC-dG). Furthermore, the binding of the enzyme to the AF-modified region appears to be irreversible under the condition of the assay which is similar to the effect of AAF adducts as described by Pohl-Leszkowicz et al. on chicken DNA. However, a much lower rate of methylation along AF-modified helices compared to similarly modified DNAs with AAF is observed, suggesting that a Z transition is not implicated.

MATERIALS AND METHODS

Poly(dC-dG) and poly(BrdC-dG) were obtained from PL Biochemicals; DNase I (DN-CL) from Sigma Chemical Co.; phosphocellulose (P-11) from Whatman; hydroxylapatite (HPT) from Bio-Rad; DEAE-Sephacel from Pharmacia; and Aca34 from LKB. [³H]-methyl-S-adenosylmethionine (10 Ci/mmol) was from ICN. N-

acetoxy-N-acetyl-2-aminofluorene 9- $[^{14}\text{C}]$ (44.3 mCi/mmol) was obtained through the NCI Chemical Repository, Mid West Research Institute. The labeled N-AcO-AAF in methylene chloride was vortex evaporated to dryness under a vacuum and then dried once more with absolute ethanol before dissolving it in a minimum volume of absolute ethanol. The reaction of $[^{14}\text{C}]$ -N-AcO-AAF with poly(dC-dG) was performed under N_2 atmosphere at 37°C in 0.01 M NaCl, 2 mM $\text{Na}_3\text{citrate}$, pH 7.5, 5% ethanol for 1 h; the carcinogen to DNA-P ratio ranged from 0.1 to 0.3. Nonreacted carcinogen was removed by two ethyl acetate, one phenol- CHCl_3 , and two ether extractions, and the polymer was recovered by ethanol precipitation. The degree of bound N-AcO-AAF was assessed by liquid scintillation counting of known quantities of reacted poly(dC-dG) and was also characterized by its ultraviolet absorption spectrum in a Cary 118 spectrophotometer (32). Both methods gave comparable results for percent modification. The poly(dC-dG) reacted with N-OH-AF in vitro was a generous gift of Dr. M. Leng. AF-poly(dC-dG) was characterized spectrally and had a 260/312 ratio of 3.2. From the calibration curve given by Spodheim-Maurizot et al. (15), the AF-poly(dC-dG) was estimated to approximate 8.5% of total bases modified equivalent to 17% modification of dG.

Preparation of Rat Liver Methyltransferase. The method for purification and characterization of the physical and catalytic properties of DMase is described elsewhere (31). Briefly, with all steps at $0-4^\circ\text{C}$, DMase was isolated from 0.63 M KCl extracts of liver nuclei, as described by Simon et al. (33). The crude methylase was subsequently purified by batch absorption on phosphocellulose and eluted at 0.6 M NaCl. This fraction was absorbed onto a hydroxylapatite column, which was developed with a linear gradient from 0.05 M KPO_4 to 0.4 M KPO_4 , pH 7.5. A single peak eluting at approximately 0.18 M KPO_4 , pH 7.5, was dialysed and chromatographed on DEAE-Sephacel equilibrated in 0.02 M Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 10% glycerol (Buffer A) + 0.025 M NaCl. A linear gradient to Buffer A + 0.3 M NaCl was developed, and a single peak eluting at approximately 0.08 M NaCl was concentrated onto a small hydroxylapatite column and recovered in a minimum volume of 0.25 M KPO_4 , pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol. Fraction III enzyme was further purified by gel filtration on AcA 34 column (1.5 x 90 cm) equilibrated in Buffer A + 0.1 M NaCl. Glycerol was added to 50% v/v and DMase stored in small aliquots at -20°C , or at -80°C for long-term storage. Protein concentration was determined by the method of Bradford (34) using bovine serum albumin as a standard. The DMase isolated by this procedure is about 1500-fold puri-

fied over the initial nuclear extract, has a molecular weight of 280 kDa by gel filtration, and preferentially methylated double- over single-stranded DNA and hemimethylated over unmethylated double-stranded DNA.

Methylation of DNA. The standard assay in 200 μ l consists of 3 μ Ci [3 H]-methyl-SAM (2 μ M in dilute acetic acid immediately neutralized with NaH_2PO_4 before use) and enzyme protein in an assay buffer containing of 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5 mM DTT, and 1 μ g of poly(dC-dG) or modified poly(dC-dG) template (1500 pmol of dC). The incubation is carried out at 37°C for 30 min to 2 h (the linear phase of the reaction) (see Figures 1-3). With this highly purified preparation of DMase no contaminating RNA or protein methylation was detected, nor were problems of nuclease activity detected, which permitted the omission of EDTA in the reaction. Nonetheless, the reactions were stopped by the addition of 12 μ l of 5 N NaOH followed by 10 μ l of 1 mg/ml sonicated calf thymus DNA and heating at 60°C for 15 min to remove any methylated RNA contaminants and destroy SAM. The reactions were directly precipitated with an equal volume of 20% trichloroacetic acid (TCA), 40 mM sodium pyrophosphate. After 30 min on ice the DNA was trapped by filtration on GF/C (Whatman) glass filter discs, which were washed three times with 5% TCA, twice with 95% ethanol, and oven dried at 60°C before liquid scintillation counting by standard double-labeled techniques on ^3H and ^{14}C channels (when present). Methylation is expressed as pmoles incorporated/mg protein/hr which is readily calculated from the specific activity of [^3H]-SAM, the efficiency of tritium counting, μ g protein in the assay, and recovery of DNA in zero time controls. The concentration of rat liver DMase was determined by assaying the activity in 0.02 M Tris-HCl, pH 7.4, 1 mM DTT, 1 mM EDTA under enzyme limiting conditions on double-stranded poly(dC-dG). Poly(dC-dG), a homogeneous polymer of dCpdG sites, has been found to be the methyl acceptor template of choice, since base sequence effects found in natural DNAs are obviated. Furthermore, it permits direct quantitation of kinetic parameters and the effects of template modification expressed as concentration of dCpdG sites (or modified dCpdG sites).

Spectroscopy. Circular dichroism spectra were recorded using a Jasco-Durrum SP-CD instrument calibrated to 0.3133° at 290 nm using a 0.1% solution of d-10-camphorsulfonic acid (K&K Labs). The ellipticities were measured at 22°C in quartz supracil cuvettes with 1 cm optical path length. Subsequent measurements of u.v. absorption spectra were recorded in a Cary 118 with the same cuvettes. Extinction coefficients used to calculate the molar ellip-

ticity for poly(dC-dG) and poly(BrdC-dG) are 7,100 and 6,200 $\text{cm}^{-1}\text{-M}^{-1}$, respectively (35). Buffer composition is as noted in the Figure legends when different from the methylase assay buffer.

RESULTS

The alternating copolymer poly(dC-dG) in its double-stranded form was used in these studies to approximate a homogenous DNA template with respect to DMase recognition sites; thereby circumventing potential modifying effects of neighboring sequences adjacent to 5'dCpdG sites in native DNAs. In this fashion kinetic parameters with respect to the concentration of 5'dCpdG sites can be determined and compared with those after carcinogen modification. Poly(dC-dG) was reacted in vitro with N-acetoxy-AAF or N-OH-AF to very similar overall degrees of substitution of AAF or AF moieties (9.5% and 8.5 %, respectively). These templates were compared with each other and against a poly(dC-dG) control template in subsequent experiments with an in vitro methylation system that utilizes 1500-fold purified rat liver DMase.

In Figure 1 the time course of methylation of 1 μg input of these arylamine-modified templates was determined in an assay containing 1.5 units of DMase. Under these conditions the template concentration of dCpdG sites is in vast excess, and the rate of methylation is proportional to the amount of added enzyme. The modified poly(dC-dG) templates display greatly diminished capacity to accept methyl groups. A greater degree of inhibition of enzymatic methylation is observed for AF adducts (90%) than for AAF adducts (80%) for similar degrees of substitution of poly(dC-dG). We have detected this inhibitory effect by the various arylamine adducts on the DMase catalysed

Figure 1. Time course of enzymatic methylation of equal inputs of unmodified and modified poly(dC-dG) templates. (●—●), poly(dC-dG); (■—■), AAF-poly(dC-dG); (▲—▲), AF-poly(dC-dG).

methylation reaction at very low levels of modification (<0.5%)(data not shown).

We next performed competition-type experiments in which, after 30 min methylation of control poly(dC-dG), an equal mass of either AF- or AAF-poly(dC-dG) was added to the methylation system, as shown in Figure 2. The addition of either carcinogen-modified template slowed down the rate of methylation, although the amount of template was doubled. This implies that while DMase is engaged in methylating poly(dC-dG), it must undergo a dissociation from this template and commence methylation of the carcinogen-modified templates at combined rates that are intermediate between those of poly(dC-dG) and the carcinogen-modified template (Figure 1). As seen in Figure 1, AF modification of poly(dC-dG) appears to exert a greater inhibitory effect than AAF for equal amounts of competitor carcinogen-modified template.

In Figure 3 the same experiment was performed in reverse order of addition of the reaction components to determine the relative rates of DMase dissociation from the carcinogen-modified poly(dC-dG) templates. After 30 min of methylation of 1 μ g of poly(dC-dG), AAF-poly(dC-dG), or AF-poly(dC-dG) in vitro, 0.5 μ g of competitor poly(dC-dG) was added to each incubation mixture, and the time course of methylation was determined over the next hour. In the control poly(dC-dG) reaction the concentration of methylation sites in the template were already in excess, and the effect of additional poly(dC-dG)

Figure 2. Effect of competitor AF- and AAF-poly(dC-dG) templates on enzymatic methylation of poly(dC-dG). Unmodified poly(dC-dG) was methylated at 37°C in vitro under standard assay conditions. After 30 min incubation, the reaction was split into 3 portions and an equivalent amount of poly(dC-dG), (●—●); AAF-poly(dC-dG), (■—■); or AF-poly(dC-dG), (▲—▲), was added, and the time course of the reaction followed over the next 60 min.

Figure 3. Effect of poly(dC-dG) competitor on kinetics of methylation of AF- and AAF-poly(dC-dG) templates. Templates were incubated with DMase under standard assay condition and after 30 min an equivalent amount of unmodified poly(dC-dG) was added and the kinetics measured over next 60 min (appropriate closed symbols). Kinetics in absence of competitor are denoted by open symbols. (○—○), poly(dC-dG); (●—●), poly(dC-dG) + competitor; (□—□), AAF-poly(dC-dG); (■—■), AAF-poly(dC-dG) + competitor; (△—△), AF-poly(dC-dG); (▲—▲), AF-poly(dC-dG) + competitor.

barely increased the rate. However, the rates of methylation for AAF- and AF-poly(dC-dG) are about 20% and 10% of control, respectively, and by the addition of poly(dC-dG) the rate would be expected to increase in proportion to the degree that DMase is liberated to methylate the active competitor template. Thus, if by 1 hour half of the enzyme had dissociated from the modified poly(dC-dG) template, the overall rate of methylation would be expected to increase to between 50% and 60% of the rate of control methylation. In Figure 3 the effect of late addition of poly(dC-dG) has no effect on increasing the rate of methylation of AF-poly(dC-dG), whereas for the AAF-poly(dC-dG) it is restored by approximately 5%-10%. These results suggest that the effect of arylamine adducts in DNA are to impair the mechanism by which DMase moves along DNA strands or between templates. In the case of AF adducts, the dissociation reaction appears to be completely blocked but only partially blocked for AAF adducts in poly(dC-dG).

Since it might be expected that the interaction of DMase with a region containing arylamine adducts, which block the dissociation or movement of the enzyme, might be detected as a strong binding complex, the following experiment was performed to examine the nature of this binding. The effect of salt on the stability of the DMase/DNA complex which is formed at 37°C in the absence of salt has been described by Drahovsky and Morris (36), and interpreted on the basis that the enzyme "walks" on DNA. The DMase preparation

Figure 4. Effect of salt and preincubation on the activity of DMase on poly(dC-dG) and AF- and AAF-modified poly(dC-dG) templates. Preincubation was carried out by the addition of template to DMase for 3 min at 37°C prior to the addition of salt (open symbols). Closed symbols denote absence of preincubation, e.g., salt added to template before enzyme at 37°C. (●—●), poly(dC-dG); (○—○), poly(dC-dG) preincubated; (■—■), AAF-poly(dC-dG); (□—□), AAF-poly(dC-dG) preincubated; (▲—▲), AF-poly(dC-dG); (△—△), AF-poly(dC-dG) preincubated.

used in these experiments exhibits this classical response on single-stranded DNA and to a lesser degree on double-stranded *M. luteus* DNA (31). In Figure 4 we examine the perturbing effects of salt on the stability of such a complex with control poly(dC-dG) and compared with AF- and AAF-modified poly(dC-dG). By preincubation of DMase with the template for 5 min at 37°C before the addition of salt, a significant stabilization is observed when compared to the addition of salt to the template before incubation with DMase. This results in a curve shifted to the right, showing an enhanced stability with a maximum activity at 25 mM NaCl for this preformed enzyme/DNA complex. When these curves are determined for AAF-poly(dC-dG) and AF-poly(dC-dG), the general shape and displacement of the preincubation curves are similar, although the initial methylation acceptor levels are much lower on these templates. This suggests that the component of ionic binding between DMase and the various sites on unmodified or carcinogen-modified template are not significantly different. Thus, the lower degree of dissociation on arylamine-modified templates is probably due to increased hydrophobic interactions in the binding of enzyme to arylamine moieties on the DNA.

In Figure 5 the kinetics of methylation of these templates was determined with respect to the total concentration of dCpdG units in the polymers. The

Figure 5. Lineweaver-Burk plots of kinetics of enzymatic methylation of modified and control unmodified poly(dC-dG) with respect to dinucleotide concentration. (○—○), poly(dC-dG); (■—■), AAF-poly(dC-dG); (▲—▲), AF-poly(dC-dG).

curves give single-value Michaelis-Menten parameters, with relative apparent $V_{\max}$ of reaction of 12.5, 3, and 0.83 pmole/hr for control, AAF- and AF-modified poly(dC-dG), respectively. If we normalize the control to unity the relative velocities are 0.24 and 0.066 for AAF- and AF-poly(dC-dG). The apparent k_m 's for the reactions are 10 μM for unmodified and AAF-poly(dC-dG), but is three-fold lower, 3 μM for AF-poly(dC-dG), which suggest a higher affinity for the latter modified DNA, but no change for the former.

In Figure 6 the CD spectra are shown for the 8.5% substituted AF-poly(dC-dG) under various conditions. In the DMase assay buffer used in this study (20 mM Tris-HCl, pH 7.4, 0.5 mM DTT, and 50 mM NaCl), the CD spectrum of AF-poly(dC-dG) exhibits a slight difference in the CD band centered on 290 nm, in which the extremum is reduced from $\Delta\epsilon$ of $1.6 \text{ M}^{-1}\text{cm}^{-1}$ to approximately $0.95 \text{ M}^{-1}\text{cm}^{-1}$. The rest of the CD spectrum is conserved and typical of the B-form of poly(dC-dG), with extremum at 276 nm, cross-over at 257-258 nm, and negative extremum at 240 nm. In 4 M NaCl, the AF-poly(dC-dG) spectrum adopts an inverted profile that, however, does not fully develop the negative extremum at 292 nm characteristic of poly(dC-dG) in Z form having $\Delta\epsilon$ of $-4.4 \text{ M}^{-1}\text{cm}^{-1}$. For comparison, we also dialysed poly(dC-dG) against a methylase assay buffer containing 2 mM MnCl_2 . The resultant CD spectrum has an irregular positive extrema in the low energy end however, upon heating to 60°C it adopts a spectrum characterized by a smaller maximum at 292 nm than in Z, termed Z*, as shown by van de Sande and Jovin (37). These results suggest

Figure 6. Circular dichroism spectra of AF-poly(dC-dG), (—●—); and AF-poly(dC-dG) containing 4 M NaCl, (●●●●); all in methylase assay buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5 mM DTT). Poly(dC-dG) in B form in methylase assay buffer, (—); and poly(dC-dG) in Z form in 4 M NaCl, (—); poly(dC-dG) in methylase assay buffer + 2 mM MnCl₂, no heating, (●●●●); same after heating at 60°C for 5 min and cooling to 22°C before recording spectra, (---).

that the AF-poly(dC-dG) conformation is not purely in B form under conditions of the assay. It does not, however, display any evidence of being in the Z conformation, except under conditions that favor a shift to the Z conformation (4 M NaCl), then the AF-poly(dC-dG) polymer appears to undergo a conformational shift to the Z form. Furthermore, the conformation of AF-poly(dC-dG) observed in the methylase assay buffer could not be altered to Z* by heating at 60°C, indicating that the molecule was not in a metastable state as might be suggested from the reduced positive $\Delta\epsilon$ at 292 nm in the AF-poly(dC-dG).

In Figure 7 the CD spectrum of the 9.5% substituted AAF-poly(dC-dG) is presented in the methylase assay buffer and the same buffer containing 4 M NaCl. The characteristic negative extremum for the Z conformation at 292 nm is decreased from $\Delta\epsilon$ of -1.8 to $-2.2 \text{ M}^{-1}\text{cm}^{-1}$ by the addition of salt, suggesting that the AF-poly(dC-dG) is essentially all in the Z conformation under conditions of the assay. Sage and Leng have shown that derivatization of poly(dC-dG) with AAF greatly facilitated the B $\rightarrow$ Z conformational drift, while AF only slightly facilitated this conversion (17). This conversion of poly(dC-dG) by AAF modification to the Z form is dependent upon the degree of derivatization, but also the concentration of monovalent counter ions, and the

Figure 7. Circular dichroism spectra of AAF-poly(dC-dG) in methylase assay buffer, (—●—); AAF-poly(dC-dG) in methylase assay buffer containing 4 M NaCl, (•••••); poly(dC-dG) 0% modification in methylase assay buffer, B form, (—); poly(BrdC-dG) in methylase assay buffer, Z form independent of salt, (---).

presence of either divalent cations or organic solvents (19). In the case of the present arylamine substituted polymers, their CD spectra were unaffected by the addition of EDTA to 1 mM or the addition of enzyme alone (data not shown). This precludes the possibility that any stabilization of these polymers was due to divalent cations or interaction with DMase in this study, but is instead intrinsic to the modification caused by the carcinogen.

DISCUSSION

Deoxyguanosine residues in DNA are the most highly susceptible to covalent modification by carcinogenic alkylating agents, which form principally N(7), O(6) and N(3) alkyl adducts (38). They are also subject to a variety of modifications by other carcinogen classes: the polycyclic aromatic hydrocarbons such as benzo[a]pyrene metabolites, which form exocyclic N(2) adducts (39); and arylamine compounds such as AAF metabolites, which principally form C(8) but also exocyclic N(2) adducts (1). In mammalian genomes the major modification of dC occurs in the dinucleotide 5' dCpdG and, to a lesser extent, in the outer C of d[CCGG] (40) and d[CC] (41). Thus, although this dinucleotide is under-represented in mammalian genomes (42), the formation of

an adduct on dG has a high probability of altering a potential methylation site in several possible ways. One possibility is that the methylation site is no longer recognized as a "proper" site for methylation because of a change in configuration of the dG base. Conversely, the base configuration may not play an important role in enzyme recognition of the site, but specificity may depend upon the local template conformation. Methylation of dC or adjacent carcinogenic modification of dG may be sufficient to induce a partial or complete conformational change that the enzyme recognizes. A model for this is provided by the B $\rightarrow$ Z transition in poly(dm⁵C-dG) that can be stabilized in Z form under physiological conditions (20). Thus, agents such as AAF (17,18) or those that alkylate the N(7) of dG (43) in unmethylated d[CG] sites may either directly prevent methylation (Z-DNA is not a substrate) or signal the enzyme that the site is already methylated because of its shift in conformation.

In this study we were interested in testing these possibilities using the model template poly(dC-dG) which undergoes B $\rightarrow$ Z conformational shifts with certain carcinogen modifications. Under ionic condition shown to stabilize poly(dm⁵C-dG) in Z form, we methylated in vitro poly(dC-dG) that had been modified to similar degrees with AF or AAF moieties. The efficiency of these templates to accept methyl group transfer is reduced by 80% to 90% for approximately one dG in 16 modified. It is clear that arylamine modification inhibits the step involving translocation from one site to the next as the rate of methylation is drastically reduced. In competition experiments, this translocation which involves dissociation and rebinding to another template is blocked. This effect has already been demonstrated by Pfohl-Leszkowicz et al. (30) for AAF modification of chicken DNA. In our study we find that AF modification produces a greater inhibition in rate of methyl group transfer and inhibition in translocation or dissociation on DNA than AAF modification for similar degrees of substitution. An examination of the enzyme/DNA complex under conditions of preincubation with or without salt suggests that the impaired translocation is not a manifestation of increased ionic attraction but rather hydrophobic interaction involving the fluorene ring.

Studies on model templates have shown that the conformation of AF and AAF adducts to dG residues are markedly different (14,15,17-19). The absence of the acetyl group permits the fluorene ring to be partially buried in the major groove of B-DNA, and thus making it perhaps more accessible to interaction with DMase. On the other hand, the acetyl group sterically interferes with the normal anti configuration and induces a syn conformation. This leads to

either ring insertions into the double helix and dG lying on the outside [insertion-denaturation model (44)], or a base displacement, in which the base pairing of the modified dG with the opposite dC is broken (14). In alternating stretches of d[CG] the syn conformation in dG and the anti conformation in dC is favorable for the sequence to adopt the Z conformation. In a left-handed helix the fluorene residue bound to C(8) would lie on the outside of such a helix and, thus, also be more accessible to interaction with a protein. Our circular dichroism results indicate that under the condition of modification and salt used in the DMase assay, AAF-poly(dC-dG) is in Z form, and the fluorene ring is probably on the outside of the helix and can interact with DMase, as shown by the impaired dissociation observed in competition experiments.

Based upon the expected properties of left-handed DNA, it is quite surprising that the enzyme recognizes and methylates AAF-poly(dC-dG) in the Z conformation, albeit at much reduced rates. Yet, it has been noted that RNA polymerase can function quite well on DNA in the Z* conformation, another closely related family of left-handed DNAs (37). Recently, Phofl-Leszkowicz et al have demonstrated that poly(dC-dG) containing 7-methyl dG modification could be methylated as well as the native B form when induced into the Z conformation (45). From our results with the two types of adducts, we believe that the major effect of arylamine covalent modification in altering enzymatic methylation hinges upon the configuration of the dG base, which must be faithfully recognized. We have noted very similar effects on methylation by anti-benzo[a]pyrene diol epoxide adducts in poly(dC-dG), which do not destabilize the helix or facilitate a B $\rightarrow$ Z transition (31,46).

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ABBREVIATIONS: AAF, N-acetyl-2-aminofluorene; AF, 2-aminofluorene; N-OH-AAF, N-hydroxy-N-acetyl-2-aminofluorene; N-OH-AF, N-hydroxy-2-aminofluorene; N-AcO-AAF, N-acetoxy-N-acetyl-2-aminofluorene; CD, circular dichroism; DMase, DNA methyltransferase; SAM, S-adenosylmethionine; TCA, trichloroacetic acid.

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