Induction of the Z conformation in poly(dG-dC)-poly(dG-dC) by binding of N-2-acetylaminofluorene to guanine residues

(circular dichroism/nuclease S1/chemical carcinogen)

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ABSTRACT Poly(dG-dC)-poly(dG-dC) and poly(dG)-poly(dC) were modified by treatment with N-acetoxy-N-2-acetylaminofluorene, and their conformations were examined by circular dichroism and susceptibility to nuclease S1 digestion. A sample of poly(dG-dC)poly(dG-dC) modified to an extent of 25% with acetylaminofluorene (AAF) at the C(8) position of the deoxyguanosine residues showed a circular dichroism spectrum that had the characteristics of the Z conformation seen in unmodified poly(dG-dC)poly(dG-dC) at high ethanol or salt concentrations. A sample of poly(dG-dC)poly(dG-dC) modified only 3% by AAF showed a spectrum characteristic of the B form of DNA. However, it was converted to the Z form at ethanol concentrations lower than required to convert unmodified poly(dG-dC)poly(dG-dC) from the B to the Z form. Poly(dG)poly(dC), which does not undergo the B-to-Z transition at high ethanol concentrations, did not show any large conformational changes with high AAF modification. Susceptibility to digestion with nuclease S1 also suggested differences in the conformations of the two modified polynucleotides. Poly(dG-dC)poly(dG-dC) modified by AAF to an extent of 25% was almost completely resistant to nuclease S1 digestion. However, both poly(dG)poly(dC) and DNA modified to similar levels by AAF were highly susceptible to nuclease S1 digestion. Two different conformations for AAF-modified deoxyguanosine are proposed, depending on whether its position is in alternating purine-pyrimidine sequences or in random-sequence DNA.

There is considerable interest in the molecular details of the covalent binding of activated derivatives of chemical carcinogens to DNA because it appears that these reactions constitute critical events in the process of carcinogenesis (1). The reaction of N-acetoxy-N-2-acetylaminofluorene (N-AcO-AAF) at the C(8) position of the deoxyguanosine residues in native DNA presents certain steric problems because, in the B conformation of DNA, the anti conformation of these residues, which is characteristic of helices that have Watson–Crick geometry, creates considerable crowding at this position. Evidence has been obtained that this reaction is associated with a distortion of the DNA helix, termed base displacement, in which the acetylaminofluorene (AAF) modified deoxyguanosine residue assumes the syn conformation by rotation of the guanine base about the glycosyl bond. Also, it has been suggested that the AAF residue is inserted into the helix perpendicular to the helix axis so that it occupies the position formerly occupied by the displaced guanine residue (2, 3). In helices that have Watson–Crick geometry, the deoxyxynucleosides are all in the anti conformation. However, a new, double-stranded conformation of deoxyxynucleic acids, designated Z-DNA, has recently been described in which the deoxyxynucleosines are in the syn conformation (4). There is evidence that Z-DNA can occur in both crystal form and solution. The crystal structure of the hexanucleotide d(C-G)₄ has been shown to be an anti-parallel double-helical fragment that has Watson–Crick base pairing (4). Unlike B-DNA, the structure has a left-handed rather than a right-handed helical sense. Neighboring segments pack on top of one another to approximate an infinite helix that has 12 base pairs per turn. The conformations of the guanine and cytosine deoxynucleotides differ, so that the helical repeating unit is made of dinucleotides rather than the mononucleotides seen in B-DNA. The torsion angles of the bases relative to the sugars are different. Cytosine residues adopt the anti conformation relative to deoxyribose, as in B-DNA, but guanine residues adopt the syn conformation.

In addition to these single-crystal results, x-ray fiber analysis of poly(dG-dC)poly(dG-dC) shows similar helical parameters, 12 base pairs per turn, and evidence for a dinucleotide repeat unit (5). Similar left-handed conformations have been observed with poly(dA-dC)poly(dG-dT) and poly(dAs⁺T)poly(dAs⁺T). Left-handed helical conformations are thus not confined to poly(dG-dC)poly(dG-dC), but also occur in DNA that has alternating purine-pyrimidine nucleotide sequences, as suggested by Wang et al. (4).

Previous studies by Pohl and Jovin (6) on poly(dG-dC)poly(dG-dC) have shown that there are two distinct forms in solution and a cooperative transition between them that occurs at high salt concentrations. The circular dichroism (CD) spectrum of the high-salt form is virtually an inversion of that of the low-salt (B-DNA) form. Similar changes in the CD spectrum were seen at high ethanol concentrations (7). In addition, an NMR study of d(C-G)₄ by Patel et al. (8) has suggested that the repeat unit at high salt concentrations could be a dinucleotide. Two peaks appeared in the high-salt [³¹P]NMR spectrum, suggesting differences between the two phosphodiester linkages.

It is not yet known whether Z-form DNA occurs in vivo; thus, its biological significance is uncertain. However, numerous stretches of alternating purine-pyrimidine sequences that have the potential to form Z-DNA exist in naturally occurring nucleic acids (9). If this type of structure does occur, it may have important consequences relative to carcinogen binding. As a direct consequence of the syn conformation of the deoxyguanosine residues in Z-DNA, the C(8) position is exposed on the outer surface of the DNA molecule. The reactivity of these positions may therefore be increased. Mutational "hot spots" have been shown to exist in the histidine D gene of Salmonella, which has a segment of eight alternating C-G residues (10). It is also possible that, if certain DNA sequences are in a dynamic equilib-

Abbreviations: AAF, acetylaminofluorene; N-AcO-AAF, N-acetoxy-N-2-acetylaminofluorene; CD, circular dichroism.
rium between the B and Z forms, modification by AAF or certain other carcinogens could "lock" these sequences into the Z form. If this occurred in vivo, it might markedly alter the biological properties of these regions of the genome.

We have investigated the susceptibility of poly(dG-dC)poly(dG-dC) to carcinogen binding under conditions in which it can exist in either the B or Z form. In addition, we have examined possible conformational changes in poly(dG-dC)poly(dG-dC) that result from increasing extents of AAF modification, by using both CD and susceptibility to nuclease S1 digestion as probes of nucleic acid conformation. Our preliminary findings have been reported elsewhere (11). While this work was in progress, a report appeared by Sage and Leng (12) dealing with some of the effects of AAF modification of poly(dA-dC)poly(dG-dC).

MATERIALS AND METHODS

Poly(dG-dC)poly(dG-dC) and poly(dG)poly(dC), and native calf thymus DNA were treated with N-AcO-AAF in 1 mM phosphate buffer (pH 7.5), unless otherwise stated. For the synthetic polynucleotides, 0.25 mg of material was incubated with 0.2 mg of [14C]AAF (750 μCi/mmoll or unlabeled N-AcO-AAF in 2 ml of buffer. For the calf thymus DNA samples, 1 mg was incubated with 0.75 mg of N-AcO-AAF. After incubation for 3 hr at 37°C, the unbound carcinogen was removed by extraction with ether. The extent of modification was determined from the ratio A300:A260 as described (13) or from the amount of bound radioactive activity. Hydrolysis of the modified polymers was carried out enzymatically, and the carcinogen deoxynucleotide adducts were isolated on Sephadex LH-20 as described (14). High-pressure liquid chromatography analysis was carried out on a DuPont model 850 instrument, using Waters μBondapak C18 columns and a water/methanol gradient.

CD studies were carried out on a Cary model 60; samples were 0.05–0.1 mM in nucleotide. Molar ellipticities ([θ]) were calculated per nucleotide residue.

Nuclease S1 digestion of [14C]-labeled AAF-modified DNA and synthetic polynucleotides was carried out essentially as described by Vogt (15). Reaction mixtures contained polymer at 0.05 mg/ml, 30 mM sodium acetate buffer (pH 4.5), 50 mM NaCl, 1 mM ZnSO4, and nuclease S1 at 2500 units per ml and were incubated at 37°C. One-milliliter fractions were removed at various times, and 0.1 mg of native DNA was added as carrier, followed by 2 ml of ice-cold 10% perchloric acid. After 15 min on ice, the solutions were filtered through glass fiber filters (Whatman GF/A). The percentage of digestion was calculated from the A260 of the filtrate, correcting for the 40% hyperchromicity associated with hydrolysis, and also from the radioactivity present in aliquots of the filtrate.

RESULTS

To determine the type and quantity of AAF adducts formed in poly(dG-dC)poly(dG-dC) under conditions in which it is in the B or the Z form, the polymer was modified with N-[14C]AcO-AAF in the presence of various concentrations of ethanol or MgCl2. Previous studies (6, 7) have provided evidence that, in 25% ethanol, poly(dG-dC)poly(dG-dC) is in the B form while, in 55%, it is in the Z form. We found that the extent of covalent binding in 55% ethanol was less than one-tenth of that in 25% ethanol (Table 1). Similarly, modification at high Mg2+ concentrations resulted in AAF binding levels that were about one-tenth of that at low Mg2+ concentrations. Because we expected that the exposure of the C(8) position of the guanine residues on the exterior of a double helix in the Z form would give higher levels of modification, we examined whether the low extents of modification obtained at high ethanol or Mg2+ concentrations are related to conformational differences in the nucleic acid or simply to differences in chemical reactivity related to the solvent conditions. As a control, calf thymus DNA was modified under the various solvent conditions and the AAF binding levels were determined. As with poly(dG-dC)poly(dG-dC), we found a marked decrease in AAF binding at either high ethanol or high Mg2+ concentrations. Although the CD spectrum of calf thymus DNA is known to change slightly under these conditions (16), it is not converted to that of the Z form. Therefore, the decreased AAF binding seen in 55% ethanol or 1 M MgCl2 is not due to steric aspects associated with the Z conformation but rather to a decrease in chemical reactivity of N-AcO-AAF. Consistent with this conclusion were additional studies suggesting that the interaction of free guanine with N-AcO-AAF is also markedly inhibited in solutions having high concentrations of ethanol or MgCl2 (data not shown).

Identification of the types of AAF adducts formed when N-AcO-AAF reacted with poly(dG-dC)poly(dG-dC) was obtained by enzymatic hydrolysis of the modified polymer to deoxyribonucleosides followed by chromatography on Sephadex LH-20; the material eluted by ethylene was further characterized by high-pressure liquid chromatography. The principal adduct (>95%) formed when both the Z and B form of poly(dG-dC)poly(dG-dC) were modified was identified as the C(8) guanine adduct, N(deoxyguanosin-8-yl)-AAF.

CD Studies. The CD spectra of poly(dG-dC)poly(dG-dC) in 1 mM phosphate buffer and in 60% ethanol are shown in Fig. 1. As shown previously (7), the spectrum at high concentrations of ethanol is nearly an inversion of that at low ethanol concentrations. Also shown in Fig. 1 is the spectrum of poly(dG-dC)poly(dG-dC) modified to the extent of 25% with AAF. It can be seen that at this high level of modification, even in aqueous solution, the conformation of poly(dG-dC)poly(dG-dC)-AAF has a CD spectrum characteristic of Z-DNA. A separate sample of poly(dG-dC)poly(dG-dC)-AAF in which 22% of the residues were modified with AAF had a spectrum in aqueous solution similar to that of the sample with 28% modification. In contrast, a poly(dG-dC)poly(dG-dC) sample modified to an extent of only 3% had a CD spectrum in 1 mM phosphate buffer that resembled that of B-DNA (Fig. 2). With the latter sample, increasing the concentration of ethanol resulted in a transition to the Z form at about 45% ethanol, whereas unmodified poly(dG-dC)poly(dG-dC), the transition was not seen until 50–55% ethanol. Thus, although 3% modification of poly(dG-dC)poly(dG-dC) with AAF did not induce a conformation that had a CD spectrum characteristic of Z-DNA, the modified sample underwent a transition to this form at a lower

Table 1. Binding of AcO-AAF to poly(dG-dC)poly(dG-dC) and DNA

<table>
<thead>
<tr>
<th></th>
<th>25% EtOH</th>
<th>55% EtOH</th>
<th>1 M MgCl2</th>
<th>0 M MgCl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(dG-dC)</td>
<td>1.22</td>
<td>0.102</td>
<td>6.35</td>
<td>0.598</td>
</tr>
<tr>
<td>DNA</td>
<td>0.474</td>
<td>0.0619</td>
<td>1.13</td>
<td>0.175</td>
</tr>
</tbody>
</table>

*Binding is expressed as nmol of AAF per μmol of nucleotide residue.*
The digestion of dC)-poly(dG-dC) on formation of single-stranded AAF-modified sites at provided single-strand calf of SI (see Fig. 3) 17% poly(dC)-poly(dG-dC) (Fig. 3). AAF 255 at of spectra of under these conditions (6). Therefore, we examined the CD spectra of poly(dG)-poly(dC) before and after modification with AAF (Fig. 3). The unmodified sample had a positive maximum at 255 nm with a crossover at 244 nm, characteristic of poly(dG)-poly(dC) (17, 18). A minor positive peak observed at 290 nm presumably reflects a small amount of single-stranded regions due to excess poly(dC) (18). In contrast to the results obtained at a high AAF modification of poly(dG-dC)-poly(dG-dC) (see Fig. 2), the spectrum of poly(dG)-poly(dC) modified to 17% with AAF was almost identical to that of unmodified poly(dG)-poly(dC) (see Fig. 3) in 1 mM phosphate buffer.

**Nuclease S1 Digestion.** Previous studies on the susceptibility of AAF-modified calf thymus DNA to digestion by nuclease S1, a single-strand specific endonuclease from Aspergillus oryzae, have provided evidence for localized regions of denaturation at sites of AAF modification (14, 19). The kinetics of hydrolysis of AAF-modified DNA is intermediate between that of native and of fully denatured DNA, suggesting that AAF binding generates single-stranded regions (14, 19). To obtain additional information on the conformations of AAF-modified poly(dG-dC)-poly(dG-dC) and poly(dG)-poly(dC), their susceptibilities to nuclease S1 digestion were investigated. The percentages of digestion of various modified and unmodified nucleic acids, based on units of A₂₆₀ absorbance or cpm found in the filtrate after perchloric acid precipitation are shown in Table 2. As expected, heat-denatured calf thymus DNA was almost completely hydrolyzed after a 2-hr incubation with nuclease S1, and native DNA was quite resistant to nuclease S1 digestion. In agreement with previous results (14, 19), ¹⁴C-labeled AAF-modified DNA showed considerable susceptibility to nuclease S1; a sample modified to an extent of 20% showed 75–83% digestion. These results are in sharp contrast to those obtained with the poly(dG-dC)-poly(dG-dC) samples. Both unmodified poly(dG-dC)-poly(dG-dC) and poly(dG-dC)-poly(dG-dC) modified with AAF to an extent of 28%, a level that results in almost complete nuclease S1 digestion of AAF-modified DNA, were almost completely resistant to nuclease S1 digestion (Table 2). Poly(dG)-poly(dC), which cannot assume the Z conformation (6), showed different results: unmodified poly(dG)-poly(dC) was quite resistant to nuclease S1 digestion but a sample with 19% AAF modification was very susceptible to digestion [54% of the radioactivity was rendered acid soluble under our standard conditions (see Table 2)].

**DISCUSSION**

Our data provide evidence that AAF modification of poly(dG-dC)-poly(dG-dC) favors the transition of the polymer from the B to the Z conformation. Previous studies with unmodified poly(dG-dC)-poly(dG-dC) suggested that this transition can be induced by high ethanol or salt concentrations because these solvents induced an inversion of the CD spectrum (6, 7). In the
The present studies, we obtained CD evidence that samples of poly(dG-dC)poly(dG-dC) that had been highly modified with AAF (20–30%) exist in the Z conformation, even in 1 mM phosphate, and that samples having a lower extent of AAF modification (3%) are induced to undergo the B-to-Z transition at a lower ethanol concentration than that required to induce the transition in unmodified poly(dG-dC)poly(dG-dC). The recent report by Sage and Leng (12) has also shown that poly(dG-dC)poly(dG-dC) modified by AAF to the extent of 6–8% undergoes a CD transition at lower ethanol concentrations than unmodified poly(dG-dC)poly(dG-dC). In contrast to the results obtained with poly(dG-dC)poly(dG-dC), we found that extensive AAF modification of poly(dG-dC)poly(dG-dC), which cannot adopt the Z conformation because it lacks alternating purine-pyrimidine sequences, had a CD spectrum that was similar to that of unmodified poly(dG)poly(dC). Mercado and Thomasz (17) have described CD changes induced by mitomycin C modification of poly(dG-dC)poly(dG-dC) that are similar to those that we observed as a result of AAF modification. They also found that these CD changes were not seen in native RNA or poly(dG)poly(dC) modified with mitomycin C (17). Although the precise structure(s) of mitomycin C–nucleic acid adducts are not known, these results suggest that mitomycin C modification of poly(dG-dC)poly(dG-dC) also favors the transition from the B to the Z form.

It is likely that some type of equilibrium exists between the B and the Z conformations in poly(dG-dC)poly(dG-dC). This was suggested by the initial crystallographic studies (4) in which the double-stranded hexanucleotide d(C-G)$_3$ crystallized out of a low-salt buffer in which B-DNA is the stable form yet produced Z-DNA crystals. Probably the crystals were nucleated as Z-DNA and, as they grew, the equilibrium shifted. In the present studies, AAF modification of the C(8) position of guanine stabilizes the molecule in the Z conformation. It is likely that this stabilization has considerable specificity.

The present results, taken together with previous studies (2, 3, 30, 21), suggest that AAF modification of the C(8) position of guanine residues in nucleic acids can be associated with at least two alternative conformations. With poly(dG-dC)poly(dG-dC), and perhaps other alternating purine-pyrimidine sequences that can assume the Z conformation, AAF modification enhances conversion to the Z conformation. In this case, there is little or no denaturation of the guanine–cytidine base pairing; we found that AAF-modified poly(dG-dC)poly(dG-dC) remains resistant to nuclease S1 digestion. In this conformation, we assume that the AAF residue is not inserted into the DNA helix, although the precise orientation of the AAF residue in Z-DNA requires further study. For nucleic acids that cannot assume the Z conformation [e.g., poly(dG)poly(dC) or random sequences in native DNA], AAF modification is associated with disruption of the double-stranded structure, displacement from the helix of the modified guanine, and insertion of the AAF residue into the DNA helix, as described in the base-displacement model (22). In contrast to the AAF–Z-DNA conformation, the base displacement conformation for AAF–B-DNA is associated with increased susceptibility to digestion by nuclease S1 (14, 19). In a sense, the major difference between these two is in the process by which the AAF-modified deoxyguanosine residue is held in the syn conformation. In Z-DNA, this results from the unusual conformation of the phosphate-sugar backbone such that deoxyguanine residues already exist in the syn conformation whereas, in the base-displacement model, the conformation of the backbone is minimally perturbed and the syn conformation of the modified deoxyguanine residue is achieved by rotation of the guanine base about its glycosyl bond.

Although there is as yet no evidence for the existence of Z-DNA in vivo, the occurrence of stretches of alternating purine-pyrimidine sequences in certain naturally occurring DNAs (9) suggests that they could exist, particularly in chromatin structures in which the Z conformation might be stabilized by protein–DNA interactions. If regions of Z-DNA do exist in vivo, then they might be more susceptible to attack by AAF and related carcinogens for the reasons discussed above. It is true that, in the present in vitro study, we were unable to demonstrate that Z-DNA is more reactive than B-DNA toward N-AcO-AAF, but this probably reflects the unusual solvent conditions (high ethanol or salt concentrations) required to maintain Z-DNA in vitro. Our positive results suggest that if, in vivo, a region of alternating guanine–cytidine sequence in the B conformation were modified by AAF, then this would favor its transition to the Z conformation. It is conceivable that this marked change in nucleic acid conformation could induce important functional features.

**Table 2. Nuclease S1 digestion**

<table>
<thead>
<tr>
<th>Modification, %</th>
<th>Digestion, %</th>
<th>Based on</th>
<th>Based on</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>A$_{260}$</td>
<td>cpm</td>
</tr>
<tr>
<td>Native DNA</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Denatured DNA</td>
<td>0</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>AAF-modified DNA</td>
<td>5</td>
<td>68</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>75</td>
<td>83</td>
</tr>
<tr>
<td>Poly(dG-dC)poly(dG-dC)</td>
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<td>7</td>
<td></td>
</tr>
<tr>
<td>AAF-modified poly(dG-dC)poly(dG-dC)</td>
<td>5</td>
<td>7</td>
<td>4</td>
</tr>
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<td></td>
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<td>3</td>
</tr>
<tr>
<td>Poly(dG)poly(dC)</td>
<td>0</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>AAF-modified poly(dG)poly(dC)</td>
<td>19</td>
<td>59</td>
<td>54</td>
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changes in that region of the cellular genome. Further studies are required, therefore, to examine the biologic significance of the present findings.

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