Induction of the Base Displacement or Z Conformation in DNA by N-2-Acetylaminofluorenone Modification
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Modification of deoxyguanosine at the C8 position by the carcinogen N-acetoxy-N2-acetylamino-
fluorenone (N-AcO-AAF) has been shown to result in two different conformational changes dependent
on the nucleotide sequence of the modified polymer. AAF modification of random sequence DNA re-
sults in a large distortion of the helix which is termed base displacement. In this conformation, the
carcinogen is inserted into the DNA perpendicular to the helix axis with the guanosine displaced to
the outside. Large single-stranded regions are generated which are susceptible to S1 nuclease diges-
tion and react with anti-cytidine antibodies.

A different conformation has been observed when the alternating pyrimidine copolymer, poly(dG-dC)·poly(dG-dC) is modified. At a modification level of 28% this polymer shows a CD spec-
trum characteristic of the left-handed Z-DNA seen in the unmodified polymer at high ethanol or salt
concentrations. Base pairing of the modified polymer remains intact as demonstrated by its resis-
tance to digestion with S1 nuclease and lack of reactivity with anti-cytidine antibodies.

Modification of poly(dG-m5dC)·poly(dG-m5dC) with AAF was also shown to induce the Z confor-
mination. However, for this polymer, inversion of the CD spectrum takes place at a much lower modifica-
tion level (10%) than for the nonmethylated polymer (>20%). This polymer is also resistant to S1, nu-
clease digestion consistent with its adoption of the Z conformation with AAF modification. A possible
role in gene expression for the Z conformation of AAF modified regions is discussed.

Introduction

The reaction of the carcinogen N-acetoxy-N2-acetylamino-
fluorenone (N-AcO-AAF) at the C8 position of
deoxyguanosine residues in native DNA has been
shown to result in large conformational changes in the
polymer which is expressed in a base displace-
ment model (1). In this model, the attachment of
AAF results in a change in glycosidic N3-C' bond
from the anti conformation of nucleosides with
Watson-Crick geometry to the syn conformation.
The AAF residue is inserted into the helix and
stacked with the adjacent base while the guanine
residue is displaced to the outside of the helix. A
similar model called insertion-denaturation has been
proposed by others (2). The result is a marked dis-
tortion of the double-stranded helix at the sites of
AAF modification and generation of local regions of
denaturation. This has been shown by the increased
susceptibility of AAF-modified DNA to digestion by
S1 nuclease, a single strand specific endonuclease (3,
4). A stereoscopic view of the base displacement
model is shown in Figure 1.

Recently, a new family of left-handed Z helical
structures has been described (6-7) (Fig. 2) based on
X-ray diffraction studies of alternating d(CpG)-DNA
crystals. Differences in the conformation of the
alternating deoxyguanosine and deoxycytidine resi-
dues results in a dinucleotide repeating unit, not a
mononucleotide, as in B-DNA. In Z-DNA, the deoxy-
guanosine is in the syn conformation, while the deoxy-
cytidine is in the anti conformation. The deoxy-
ribose ring of cytidine has a pucker in which the 2’
carbon is in the endo conformation, while that of
guanosine can be C3’ endo (5) or C1’ exo (7) depend-
ing on whether the sample was crystallized from low
or high salt solutions, respectively.

In solution, poly(dG-dC)·poly(dG-dC) undergoes a
Salt- (8) or ethanol-induced (9) conformational change as measured by circular dichroism (CD). The CD spectrum of the high-salt form is virtually an inversion of that of the low-salt (B-DNA) form. [32P]NMR spectra (10) show two peaks in high salts which suggest different phosphodiester linkages and a dinucleotide repeating unit. Several other studies have provided additional evidence that the high salt or ethanol solution structures correspond to the Z-DNA structure of the crystals (11, 12).

As a direct consequence of the syn conformation of the deoxyguanosine residues in Z-DNA, the C₈ position is exposed on the outer surface of the molecule (Fig. 3). Modification of this position by AAF may, therefore, not require such drastic distortion of the conformation as is seen in B-DNA where the C₈ position is crowded inside the helix. Z-DNA may, therefore, be more susceptible to carcinogen modification than B-DNA. In addition, since AAF modification of deoxyguanosine in DNA results in the rotation of the guanine from anti to the syn conformation, a similar change in conformation would be expected in poly(dG-dC)-poly(dG-dC) and might force the conformation of the entire polymer into the Z form.

Conformation of AAF-Modified Poly(dG-dC)-poly(dG-dC)

We have investigated the conformational changes induced in poly(dG-dC)-poly(dG-dC) with AAF modification (13). Figure 4 shows the CD spectra of poly(dG-dC)-poly(dG-dC) in buffer and 60% ethanol. In aqueous solution, a B-DNA spectrum is seen while in ethanol the CD is inverted and characteristic of Z-DNA. Also shown is the CD spectrum of a sample modified to the extent of 28% with AAF. With this high level of modification, the CD spec-
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FIGURE 2. van der Waals side views of Z-DNA and B-DNA. The irregularity of the Z-DNA backbone is illustrated by the heavy lines which go from phosphate to phosphate residues along the chain. The minor groove in Z-DNA is quite deep, extending to the axis of the double helix. In contrast, B-DNA has a smooth line connecting the phosphate groups and two grooves, neither one of which extends into the helix axis of the molecule. From Wang et al. (5) with permission.

FIGURE 3. End views of the regular idealized helical forms of Z and B-DNA. Heavier lines are used for the phosphate-ribose backbone. A guanine-cytosine base pair is shown by shading. The difference in the positions of the base pairs is quite striking: they are near the center of B-DNA but at the periphery of Z-DNA. From Wang et al. (6) with permission.

FIGURE 4. CD spectra of poly(dG-dC) · poly(dG-dC). In 1 mM phosphate buffer, in 60% ethanol, and modified by AAF to an extent of 28% in 1 mM phosphate buffer. From Santella et al. (13).
trum resembles that of Z-DNA. With lower levels of modification (3%), the sample still shows a CD characteristic of B-DNA, but is converted to that of Z-DNA at lower ethanol concentrations than for unmodified poly(dG-dC)-poly(dG-dC) (Fig. 5). Sage and Leng (14) have also shown that with low levels of AAF modification, the polymer undergoes the B to Z transition at lower ethanol concentrations.

To obtain additional information about the conformation of the modified polymer, its susceptibility to S, nuclease digestion was determined. Table 1 shows that AAF-modified DNA and AAF-modified poly(dG)-poly(dC) were digested and therefore contain significant single-stranded regions. In contrast, poly(dG-dC)-poly(dG-dC) modified heavily with AAF was essentially resistant to S, nuclease digestion and must be double-stranded, proving that AAF modification does not induce localized regions of denaturation in this alternating purine-pyrimidine polymer.

Denatured sites in a double-stranded polymer can also be detected by radioimmunoassay using anti-cytidine antibodies (15). These antibodies react specifically with cytidine residues which are accessible in the single-stranded regions of a polymer. They precipitate a tracer of [3H] denatured DNA (dDNA), but not native DNA (16). Addition of a competitor for reaction with the antibodies, such as nonlabeled dDNA, inhibits the precipitation of the radioactive tracer. This is shown in Figure 6, where addition of dDNA or DNA-AAF inhibits the precipitation of

Table 1. Nuclease S, digestion

| Modification, % | Digestion, % |
|----------------|-------------|
| DNA            | 0           | 0            |
| DNA-AAF        | 20          | 11           |
| Poly(dG-dC)-AAF| 28          | 19           |
| Poly(dG)·poly(dC)-AAF | 19   | 59           |

Figure 5. CD spectra of poly(dG-dC)·poly(dG-dC) modified with AAF to an extent of 3% at various ethanol concentrations: (--·--) in 1 mM phosphate buffer; (·--·) in 40% ethanol; (--) in 45% ethanol; (---·) in 50% ethanol. From Santella et al. (13).

Figure 6. Radioimmunoassay at nonequilibrium conditions in which the binding of purified anti-C antibodies to [3H]dDNA was measured in the presence of various concentrations of (●) dDNA; (○) DNA-AAF, 11% modified; (□) poly(dG-dC)·poly(dG-dC)-AAF, 21% modified; and (△) poly(dG)·poly(dC)-AAF, 5% modified. From Santella et al. (15).
the [3H]dDNA by competing for reaction with the antibodies. Poly(dG-dC)·poly(dG-dC) with a 21% modification level does not inhibit the binding of the antibodies to the tracer, which indicates that this polymer does not react with the antibodies and therefore has no significant single-strand regions. In contrast, a sample of AAF-modified poly(dG)·(dC) does react with the antibodies and thus contains some denatured regions.

**Potential Energy Calculations**

In order to propose a model for the modified polymer, potential energy calculations were performed on the dCpdG model system in collaboration with Suse Broyde and Brian Hingerty (15). A low-energy conformation whose DNA backbone is very similar to that of the dCpdG segment of Z1-DNA (6) is shown in Figure 7. The guanine is syn and approximately coplanar with cytidine, and the AAF residue is twisted nearly perpendicular to the G; the deoxyribose are alternately C3' endo in guanosine and C2' endo in cytidine. This energy-mini-
tion and anti-cytidine antibodies seem to be in accord with the suggestion that the inverted CD spectra are indicative of a Z-DNA type conformation Table 2 summarizes the data and indicates two different conformations for AAF-modified deoxyguanosine, base displacement, and Z-DNA, depending on whether the modified residues are in random or alternating purine-pyrimidine sequences. In random sequence DNA, the conformation with AAF modification is best represented by the base displacement model which involves disruption of base pairing and intercalation of the AAF residue. This partial denaturation of the DNA is detected by heat denaturation (17), increased susceptibility to digestion by S, nuclease (3, 4), and increased reactivity with anticytidine antibodies (15). The CD spectrum of AAF-modified DNA is essentially that of B form DNA (17). In contrast, alternating purine–pyrimidine sequences, when modified by AAF, adopt the Z conformation. Thus, modified poly(dG-dC) · poly(dG-dC) shows a CD spectrum characteristic of Z form DNA (15). Although the deoxyguanosine residues adopt the syn conformation, as in the base displacement model, the base pairing remains intact in Z-DNA. This is indicated by the resistance of modified poly(dG-dC) · (poly(dG-dC) to digestion with S, nuclease (13) and the lack of reactivity with anticytidine antibodies (15).

**Conformation of AAF-Modified Poly(dG-m5dC) · poly(dG-m5dC)**

The dinucleotide sequence m5dC-dG occurs frequently in eukaryotic DNA and in many organisms it comprises more than half of all dCpdG sequences. Furthermore, the presence of methylated sites within a structural gene has been implicated in the inhibition of transcription of certain eukaryotic genes (18). Recently it has been shown that the methylated polynucleotide, poly(dG-m5dC) · poly(dG-m5dC) undergoes a transition from B to Z form at much lower salt concentrations than required to convert the nonmethylated form (19). The Z form of this polymer has thus been shown to be stable under typical physiological conditions.

We have investigated the conformational changes of poly(dG-m5dC) · poly(dG-m5dC) with AAF modification. The CD spectra of the polymer bound with various levels of AAF is shown in Figure 9. At modification levels above 6%, there begins to appear a negative band at 295 nm characteristic of Z-DNA. With 10.4% modification the polymer is completely in the Z form. Poly(dG-m5dC) · poly(dG-m5dC) undergoes a B to Z transition with increasing concentrations of Mg2+, with a midpoint at 0.6 mM Mg2+ (19), compared to 0.7 M for the nonmethylated polymer. With low levels of AAF modification, it is possible to decrease further the amount of Mg2+ needed to induce the B to Z transition. A sample of poly(dG-

| Properties                  | DNA                        | [Poly (dG-dC) · poly(dG-dC)] |
|-----------------------------|----------------------------|-----------------------------|
| Conformation                | Base displacement          | Z-DNA                       |
| CD spectra                  | B-DNA type                 | Inverted B-DNA             |
| Base pairing                | Disrupted                  | Intact                      |
| Nuclease S, susceptibility  | Sensitive                  | Resistant                   |
| Anticytidine antibodies     | Reactive                   | Nonreactive                 |

**Figure 9.** Circular dichroism spectra of poly(dG-m5dC) · poly(dG-m5dC) in 50 mM NaCl, 50 mM Tris pH 8.0, and with various levels of AAF modification (---) control, (--) 6.5% modification, (••••) 8.8% modification, (•••••) 10.4% modification.
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m\(^d\)C \cdot \text{poly}(dG-m\(^d\)C) with 3.5% AAF modification has a midpoint of transition at 0.3 \(mM\) Mg\(^{2+}\) (Fig. 10). Thus both methylation at the C5 of cytosine and AAF modification at C8 of guanosine favor induction of the Z conformation.

The susceptibility of this modified polymer to S\(_1\) nuclease was also investigated. Figure 11 shows the level of digestion of the various polymers with time. Heat-denatured DNA is completely hydrolyzed by S\(_1\) nuclease while native DNA is quite resistant. A DNA sample modified to a level of 8.5% showed about 50% digestion after 3 hr. In contrast, a sample of poly(dG-m\(^d\)C) \cdot poly(dG-m\(^d\)C) with 17.5% modification shows only 14% digestion after 3 hr. incubation with S\(_1\) nuclease. These results are similar to the resistance of modified poly(dG-dC) \cdot poly(dG-dC) to S\(_1\) nuclease digestion, and indicate that base pairing also remains intact when the methylated polymer is modified.

As a direct consequence of the syn conformation of the deoxyguanosine residues in Z-DNA, the C\(^\prime\) position is exposed to the outer surface of the DNA molecule. The reactivity of these positions may, therefore, be increased. To investigate this possibility, the reactivity of poly(dG-dC) \cdot poly(dG-dC) under

![Figure 10](image10.png)  
**Figure 10.** Circular dichroism spectra of poly(dG-m\(^d\)C) \cdot poly(dG-m\(^d\)C) modified by AAF to an extent of 3.5% in various concentrations of Mg\(^{2+}\); (--) No Mg\(^{2+}\); (---) 0.2 \(mM\) Mg\(^{2+}\); (--) 0.4 \(mM\) Mg\(^{2+}\). All samples contained 50 \(mM\) NaCl, 5\(mM\) Tris, pH 8.0.

![Figure 11](image11.png)  
**Figure 11.** Nuclease S\(_1\), digestion of (●) native calf thymus DNA, (○) denatured calf thymus DNA, (□) poly(dG-m\(^d\)C) \cdot poly(dG-m\(^d\)C) modified with AAF to an extent of 17.5%, and (▲) calf thymus DNA modified with AAF to an extent of 8.5%.

![Figure 12](image12.png)  
**Figure 12.** Extent of binding of AAF to synthetic polydeoxyribonucleotides in 50 \(mM\) NaCl, 5 \(mM\) Tris, pH 8.0: (●) poly(dG \cdot poly(dC); (○) poly(dG-dC) \cdot poly(dG-dC); (□) poly(dG-m\(^d\)C) \cdot poly(dG-m\(^d\)C).
various salt and ethanol concentrations, which stabilize the B or Z conformation, was studied. Unfortunately, those conditions which induced the Z conformation, even 40 μM cobalt hexamine chloride, inhibited the reaction of N-AcO-AAF with the polymer. Instead of kinetic binding experiments, the equilibrium binding level of the various polymers with increased levels of N-AcO-AAF was determined. The results shown in Figure 12 indicate that the order of reactivity follows the ease of formation of the Z conformation: poly(dG-m5dC) · poly(dG-m5dC) > poly(dG-dC) · poly(dG-dC) > poly(dG) · poly(dC). The higher reactivity of the alternating copolymer than the homopolymer had been seen previously (20). These results seem to indicate some preferential modification of the Z form, but further studies are necessary to confirm this result.

Discussion

Of critical importance in determining the role of Z-DNA in vivo is the prior demonstration of its presence in naturally occurring DNAs. While tracts of alternating dG-dC sequences may be found, the best data, at present, is for seven base-pair lengths in the hairpin region of four related parvoviruses genomes (21). Recent studies have shown that stretches of up to 40 base pairs of dG-dC can be inserted into recombinant plasmids without interfering with their natural replication (22). These recombinants have also been shown to have both right- and left-handed conformations in the same molecule and indicates the possible role of DNA conformation in regulatory processes.

Indirect evidence for the existence of Z-DNA in vivo comes from studies using antibodies to Z-DNA (23, 24). These antibodies were shown to bind in reproducible patterns exclusively in interband regions of Drosophila melanogaster polytene chromosomes (25) which are associated with transcription of certain genes. Antibodies that react with Z-DNA were also found in the sera of mice with an autoimmune disease similar to human systemic lupus erythematosus. They occur spontaneously, but the immunogen is unknown.

A precondition for Z-DNA to be biologically active is its stabilization under physiological conditions as a left-handed segment in a mainly right-handed helix. One of the ways to achieve stabilization of Z-DNA in vivo is modification of DNA by C5 methylation of cytosine residues. Since methylation of the CpG sequence has been implicated in the inhibition of transcription of some eukaryotic genes, it is possible to assume that methylation induces the formation of Z-DNA segments which then can act as a conformational switch in the regulation of gene expression. Because AAF can also stabilize the Z-DNA conformation under physiological conditions, the proposal could be extended to chemical carcinogens such as AAF. This carcinogen could inhibit gene expression indirectly by causing a conformational switch in a region which, under normal conditions, would be transcribed. The feasibility of such a proposal is under our investigation.

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