Conformation of Oligonucleotides and Nucleic Acids Modified with 2-Amino-fluorene or 2-Acetylaminofluorene

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The first part of this work deals with the thermal stability of oligonucleotides modified with acetylaminofluorene and aminofluorene, respectively. The complementary oligonucleotides dCGCG, d(CGTCG) and d(AATTGCAATT) have been studied by ultraviolet absorption and circular dichroism. In high salt concentration and at low temperature, the three oligonucleotides form double-stranded helices which have the B-form Substitution of guanine residues in these oligonucleotides by acetylated or deacetylated aminofluorene residues destabilizes the B-form and does not induce the transition to the Z-form.

The second part of the work deals with the antibodies to Z-DNA. The specificity of these antibodies has been determined by radioimmunoassay. The antibodies react with the Z-form but not with the B-form. Poly(dG-dC).poly(dG-dC) modified by acetylaminofluorene residues is recognized by the antibodies. The antibodies can detect the Z-form in natural DNA as visualized by fluorescent staining of polytenic chromosomes from Drosophila melanogaster.

Determination of the adducts formed in the reaction between chemical carcinogens and DNA are of major importance for the understanding of tumor formation. In the in vivo and in vitro reaction between the metabolites of the hepatocarcinogen N-hydroxy-2-acetylaminofluorene (N-OH-AAF) and DNA, three major adducts were identified as N(deoxyguanosin-8-yl)-N-acetyl-2-aminofluorene (dGuo-C8-AAF), N(deoxyguanosin-8-yl)-2-aminofluorene (dGuo-C8-AAF) and 3'deoxyguanosin-N2-yl)-2-acetylamino- fluorine (dGuo-N2-AAF) (1-3). Moreover, a new adduct identified as 1-6(2,5-diamino-4-oxo-pyrimidinyl-N2-deoxyriboside)-3(2-fluorenyl) urea (rodGuo-C8-AF) has been recently found in rat liver DNA (4, 5) but not in DNA from cell culture exposed to N-OH-A AF (6).

An important factor determining excisability and persistence of DNA lesions may be their effect on DNA conformation. Bulky adducts such as those produced by benzo[a]pyrene, N-OH-AAF and aflatoxin B, cause major local distortion (7-9). Recent studies with N-acetoxy-N-acetylaminofluorene (N-OAc-AAF) suggest that adducts may also influence the conformational state of DNA over a more extended range. Substitution of guanine in poly (dG-dC)-poly(dG-dC) with acetylaminofluorene residues shifted the conformational equilibrium from the B to the Z-form (10-13) while substitution with amino-fluorene residues had no effect on the equilibrium (9).

The stabilization of the Z-form of poly (dG-dC).poly(dG-dC) by acetylaminofluorene residues led us to ask the question whether or not modified guanine residues in sequences which are not only (C-G) sequences can also stabilize the Z-form. In order to answer this question, we have undertaken a systematic study of oligonucleotides chemically modified by N-OAc-AAF. In this paper, we report results on the conformation of some modified oligonucleotides [d(CGCG)], (dCGTACG) and (dAAT-TGCAATT)] studied by ultraviolet absorption and circular dichroism. We have also compared the conformation of these oligonucleotides to those in which the dGuo-C8-AAF residues are replaced by dGuo-C8-AF.

To complement these physicochemical studies, we report an immunological study. By indirect immunofluorescence, using antibodies to Z-DNA, we confirm the presence of sequences in Z-form in polytenic chromosomes of Drosophila melanogaster.

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Material and Methods

The oligonucleotides were prepared by Dr. N. T. Thuong (Orleans) according to the phosphotriester method with several modifications (14). Chemical modification of nucleotides and oligonucleotides by N-OAc-AAF were performed as previously described with minor changes (15). In some cases, the reaction between the carcinogen and the oligonucleotides was repeated twice. After purification of the modified oligonucleotide on a Sephadex G25 column, the oligonucleotide was treated with nuclease P1 from Penicillium citrinum (EC 3.1.4) and then with alkaline phosphatase. By thin-layer chromatography (18), it has been verified that almost all if not all guanine residues were substituted.

Aminofluorene-nucleotide and oligonucleotides were prepared by alkali treatment of the corresponding acetylaminofluorene compounds (1 M NaOH, 40°C, oligonucleotide concentration ≈ 10^-4 M). The reaction was followed by absorption in a Cary 210 spectrophotometer. The reaction was stopped by addition of 1 M acetic acid in the cold, with vigorous stirring. It has been verified by thin-layer chromatography after enzymatic hydrolysis that all the dGuo-C8-AAF residues were deacetylated. A longer alkali treatment transformed dGuo-C8-AF to rodGuo-C8-AF.

Poly(dG-dC)·poly(dG-dC) was bought from PL Biochemicals. The reaction with chloroethylenetria- minoplatinum(II) chloride and the immunization of the rabbits have been previously described (16, 17), as well as the study of the specificity of the antibodies to Z-DNA (17).

The circular dichroism spectra were recorded on a Roussel-Jouan dichrograph III.

Polytene chromosomes were prepared from salivary glands of third instar Drosophila melanogaster larvae. Salivary glands were dissected out of larvae into phosphate-buffered saline, (PBS), fixed for 1 min in 45% (v/v) aqueous acetic acid and squashed under a coverslip. Dispersion of nuclei and spreading of chromosomes was checked. The preparations were then immersed in liquid nitrogen for 20 sec. After prying off the coverslip, the slides were post-fixed 1 min in 45% (v/v) aqueous acetic acid, plunged into 95% ethanol for 5 min and stored in PBS until used.

The slides were not dried between any of the washes or incubations. The chromosome spreads were covered with a PBS dilution (1:50) specific antiserum and incubated for 15 min at 37°C in a humid atmosphere. The slides were washed in PBS, and the chromosome preparations were incubated with a 1:100 dilution of fluorescein isothiocyanate (FITC) conjugated IgG fraction of sheep anti-rabbit serum (Institut Pasteur, Lot. 0374561) in PBS for 15 min at 37°C. After washing in PBS, the preparations were mounted in a drop of a solution glycine-PBS. The chromosome spreads were examined and photographed in a Leitz microscope (Dialux 20) with epifluorescence using a standard fluorescein filter and with phase contrast optics. Photographs were taken using Ilford FP4 film 125 ASA.

Results

Alkali Treatment of dGuo-C8-AAF and AAF-Oligonucleotides

It is known that deacetylation of dGuo-C8-AAF or dGuo-C8-AAF residues, occurs by alkali treatment (1, 4, 18, 19). For longer times of reaction, dGuo-C8-AF residues are transformed to rodGuo-C8-AF residues, in which the hydrolytic opening of the imidazole ring of guanine occurs through the N7-C8 bond (1, 19). The absorption spectrum of rodGuo-C8-AF residues is blue-shifted in the range of 360-310 nm as compared to that of dGuo-C8-AF residues (19, 20). The kinetics of the hydrolytic opening of the imidazole ring of AF-oligonucleotides were followed at 340 nm. The deacetylation occurred faster with the modified nucleoside than with modified oligonucleotides. On the other hand, the same kinetics were observed with modified d(CGCG), d(CGTCAG) and d(AATTGCAATT), which seems to exclude any sequence effect on the mechanism of the ring opening (results not shown).

It has been already reported that after alkali treatment of dGuo-C8-AF, two ring-opened derivatives were found. There are probably stereoisomers (19, 20). We have studied the two compounds by proton nuclear magnetic resonance (250 MHz) (results not shown), and the analysis of the spectra confirms that the opening occurs between the N7 and the C8 atoms of the imidazole ring (20). From a study with space-filling model (CPK Precision Molecular Models), we noticed a great hindrance of rotation about the N-CO bond (N-CO-NH-fluorene). The carbonyl group is blocked either on one side or the other of the 5-amino group as shown in Figure 1. This could explain why the ultraviolet absorption spectra of the two compounds are identical and the circular dichroism spectra so different (the spectrum of stereoisomer 1 is almost an inversion of the spectrum of the stereoisomer 2 (19).

Study of Modified Oligonucleotides

d(GCGG). We have studied the behavior of the tetranucleotide and this tetranucleotide remodified by AAF and AF residues by ultraviolet absorption and circular dichroism as a function of temperature. As shown in Figure 2A, the absorbance of the un-
modified tetranucleotide decreases as the temperature is decreased, which indicates the formation of an ordered structure. The circular dichroism spectrum (Fig. 2B) presents a positive band first and then a negative band. The shape of the spectrum did not change as the temperature was lowered to \(-5^\circ\text{C}\).

The modified tetranucleotide d(CGCG)-AAF also presents a cooperative transition as the temperature is decreased (Fig. 2A). The transition occurs at a higher temperature than that corresponding to the unmodified tetranucleotide. At temperature lower than 20\(^\circ\text{C}\), the solution became turbid which reflected the formation of aggregates. The circular dichroism at 36\(^\circ\text{C}\) first presents a negative band centered at 294 \(\text{nm}\), and then a positive band centered at 250 \(\text{nm}\) (Fig. 2B). This spectrum looks like the spectrum of Z-poly(dG-dC) (21). However, the circular dichroism spectrum of d(CpG)-AAF also looks like the spectrum of d(CGCG)-AAF (Fig. 3B). On the other hand, the spectrum of d(GpC)-AAF is completely different (Fig. 3B). There are two positive bands and the intensities are smaller as compared to the positive band of the spectrum of d(CpG)-AAF.

The modification of the tetranucleotide by AF residues seems to prevent the formation of an ordered structure. The absorbance at 290 \(\text{nm}\) increases slightly as the temperature is increased as shown in Figure 2C (smaller variations are observed at 270 \(\text{nm}\)). The circular dichroism presents two negative bands and then a positive one (Fig. 2D). The intensities of the bands depend upon temperature but the shape is not changed (Fig. 2D).

\(\text{d(CGTA(CG)}\)). The variation of the 260 \(\text{nm}\) absor-
bance of the hexanucleotide shows than an ordered structure is formed as the temperature is decreased (Fig. 4A). The T_m is at 30°C in 1 M NaCl, 5 mM Tris HCl, pH 7.5 (the T_m depends upon the salt and the hexanucleotide concentrations; it increases from 0.01 M to 1 M NaCl and then decreases at higher salt concentration (results not shown; see reference 14 for the effects of concentration). Even at very low temperature (−8°C), the circular dichroism spectrum presents a positive band and then a negative one (Fig. 4B).

Modifications of the hexanucleotide by AAF or AF residues do not stabilize the ordered form. In both cases, the absorbances vary slightly with temperature (Figs. 4A and 4C). This is also confirmed by circular dichroism (Figs. 4B and 4D). The shape and the intensity of the circular dichroism spectra of d(GCTAGC)-AAF and of d(GCTAGC)-AF presented only small changes as a function of temperature.

d(AATTGCAATT). The variation of the decanucleotide absorbance with temperature shows that an ordered structure is formed at low temperature which melts cooperatively as the temperature is increased (Fig 5A). The circular dichroism spectrum has a positive band first and then a negative one (Fig. 5B). It looks like the circular dichroism spectrum of B-DNA.

No evidence for an ordered structure was found with the modified AAF or AF decanucleotides by ultraviolet absorption (Figs. 5A and 5C) and by circular dichroism (Figs. 5B and 5D).

Z-Sequences in Polytene Chromosomes

Antibodies to Z-DNA were elicited in rabbits immunized with poly(dG-dC)-poly(dG-dC) modified by the reaction with chlorodithylenetraminoploutin(II) chloride (17). This polymer in which 12% of the bases were modified on the N7 of guanine residues is in Z-form in physiological conditions (16). The specificity of the antiserum was studied by radioimmunoassays. Some results in 0.1 M NaCl, 1 mM MgCl₂ and in 3 M NaCl are shown in Figure 6.

In 0.1 M NaCl, 1 mM MgCl₂, poly(dG-dC)-poly(dG-dC) is in the B-form and does not inhibit the binding of the tracer poly(dG-dC) dien-Pt to the antiserum. Poly(dG-dC)-AAF inhibits up to 100% but is less efficient than poly(dG-dC)dien-Pt. About ten times more poly(dG-dC)-AAF is necessary to get 50% inhibition. In 3 M NaCl, poly(dG-dC)-poly(dG-dC) is in
the Z-form and behaves as poly(dG-dC)dien-Pt. Poly-
dG-dC)-AAF also inhibits but now about 100 times
more poly(dG-dC)-AAF is necessary to get 50% inhi-
bition.

Polytene chromosomes from larval salivary
glands of Drosophila melanogaster were first in-
cubated in the presence of antiserum to Z-poly(dG-dC)
and then in the presence of fluorescein-labeled
sheep antibodies to rabbit immunoglobulins as
described in material and methods. The results are
shown in Figure 7. There are many fluorescent re-
gions on all the chromosome arms and dark regions.
Similar results were obtained when the experiment
was done with purified antibodies against Z-poly(dG-
dC) instead of the antiserum. The same experiment
was repeated with the serum of a rabbit which was
not immunized with poly(dG-dC)dien-Pt. The chro-
mosomes were dark.

Discussion

Several studies have been devoted to the confor-
mation of nucleic acids modified by acetyl-aminoflu-
ore and aminofluorene residues on the C8 of gua-
nine residues. Most of the results on DNA-AAF can
be explained by a local denaturation of DNA, which
has been named an insertion-denaturation model or
a base-displacement model (7, 8). However, these
models are not in agreement with the results on the
poly(dG-dC)-AAF. The covalent binding of AAF resi-
dues does not locally denature poly (dG-dC)-poly(dG-
dC) but induces the transition from the B-form to the
Z-form (10,11). In this modified alternating co-
polymer, the AAF residues are outside the double
helix, the modified guanine being paired with the
complementary cytosine. In low ionic strength,
poly(dG-dC).poly(dG-dC), in which a few guanines
are substituted by the carcinogen, behaves as a co-
polymer with sequences in B-form and sequences in
Z-form (10,11). The question was to find whether or
not modification of guanine by AAF residues in (CG)
sequences or in alternating purine-pyrimidine se-
cquences could stabilize the Z-form. To answer this
question, we have studied oligonucleotides of well-
defined sequences. Moreover, we have compared
the behavior of the AAF-modified oligonucleotides
to that of AF-modified oligonucleotides. The confor-
mation of AF-modified nucleic acid is not well
known. The covalent binding of AF residues to
poly(dG-dC).poly(dG-dC) does not induce the transi-
tion from the B-form to the Z-form (10). If in DNA-
AAF, AF residues induce a local denaturation as
AAF residues do, the size of the denatured regions
is smaller than that in DNA-AAF, as shown by the
reaction with antibodies to nucleosides (22) and with
S, nuclease (23). In fact, there is no reason to assume
a local denaturation. The study of dGuo-C8-AF and
dinucleotides-AF shows that the conformation of the
modified deoxynucleoside is anti and there is no
evidence for strong interactions between AF resi-
due and the adjacent base in the modified dinu-
cleotides (15, 24, 25). Until now, there have been no data
on the conformation of the ring-opened modified
nucleic acids.

The first part of this work is devoted to the
study of oligonucleotides modified by AAF and AF
residues. The oligonucleotides-AAF were prepared
by the reaction between the oligonucleotides and
N-OAc-AAF. The oligonucleotides-AAF were obtained
by alkali treatment of oligonucleotides-AAF. If the
oligonucleotides-AAF are kept for longer times at
basic pH, the modified guanine residues are trans-
formed in ring-opened derivatives. The kinetics of
the transformation are slower with oligonucleotides-
AF than with dGuo-C8-AF, but the three modified

![Figure 6](image1.png)

**Figure 6.** Radioimmunoassays: (A) 0.1 M NaCl, 1 mM MgCl2,
5 mM Tris-HCl pH 7.5; (B) 3 M NaCl, 5 mM Tris-HCl, pH
7.5. Percent of inhibition of tracer precipitation as a
function of the logarithm of inhibitor concentration: (●)
poly(dG-dC)dien-Pt; (○) poly(dG-dC).poly(dG-dC); (△)
poly(dG-dC)-AAF in which 13% of the bases were modified
by N-acetylamino fluorene residues. Tracer (1H) poly(dG-
dC)dien-Pt (0.12) 0.2 μM; volume of the assay 50 μL.

![Figure 7](image2.png)

**Figure 7.** Squash preparation of Drosophila melanogaster
salivary glands. Staining of Z-DNA by indirect immu-
nofluorescence.
oligonucleotides d(CGCG), d(CGTCAG) and d(AATTTGCAATT) behave similarly. There is no sequence effect on the kinetics.

By ultraviolet absorption and circular dichroism studies as a function of temperature, it can be concluded that the three complementary oligonucleotides d(CGCG), d(CGTCAG) and d(AATTTGCAATT) form double helices at low temperature, but these double helices melt cooperatively as the temperature is increased. The circular dichroism of these oligonucleotides shows that the double helices belong to the B-family (the spectrum has a positive band first and then a negative one) and not to the Z-family (the spectrum has a negative band first and then a positive one) (21). It is interesting to note that d(CG)2 is in the Z-form at low temperature and in high salt concentration (29), while d(CG)2 and d(CGTCAG) are in the B-form.

It is known that d(Guo-C8-AAF) residues are in the syn conformation (7, 8, 15). A major aim of this work was to see whether d(Guo-C8-AAF) residues in these three modified oligonucleotides could stabilize the Z-form. This is not the case with d(CGTCAG)-AAF and d(AATTTGCAATT)-AAF. This can be concluded from the variation of the absorbances as a function of temperature. The shape of the curves looks like the noncooperative melting of single-stranded oligonucleotides. AAF residues not only do not stabilize the Z-form but also destabilize the B-form. The analysis of the behavior of d(CGCG)-AAF is not so straightforward. A cooperative transition occurs at higher temperature than that of d(CGCG). However, the complete transition cannot be observed because the solution becomes turbid as the temperature is decreased below 20°C. At 36°C, the circular dichroism signal is intense but looks like the signal of d(CpG)-AAF. It seems likely that d(CGCG)-AAF can form an ordered structure at low temperature, but we do not know whether a Z-structure is formed.

The variations of the ultraviolet absorption of AF-modified oligonucleotides as a function of temperature are small and correspond to a noncooperative melting of a single-stranded oligonucleotide. The variations of the circular dichroism are also small. Thus we can conclude that the substitution of guanine residues by AF residues decreases the interactions between the modified guanines and the other bases. In our experimental conditions, it was not possible to form double-stranded helices. This is in agreement with previous results on DNA-AF. The $T_m$ of DNA-AF was lower than that of the unmodified DNA (27). This does not mean that in DNA-AF, AF residues locally denature the double helix. These modified guanines decrease the thermal stability but they can still be paired with the complementary cytosines.

These results show that in an alternating hexanucleotide, the substitution of a CpG doublet by a TpA doublet stabilizes the B-form versus the Z-form. Substitution of guanine by AAF residues which stabilize the syn conformation is not sufficient to induce the Z-form. The next question was to determine whether or not the Z-form could be only observed in very long (CpG) sequences. Furthermore, is it possible to find Z-sequences in natural DNA or is it possible to induce the Z-form by the reaction of DNA with N-OAc-AAF?

A very efficient technique to detect a structure in situ is by an immunological method. Unlike B-DNA, Z-poly(dG-dC) is a strong immunogen in rabbits (17, 28). We have induced the synthesis of antibodies against Z-DNA in rabbits by immunization with poly(dG-dC)·poly(dG-dC), in which about 12% of the bases are modified by chloroethylendiaminetetraaminoplatinum(II) chloride. The antisera reacts with the Z-form and not with the B-form. Substitution of guanine residues in poly(dG-dC)·poly(dG-dC) by AAF residues decreases but does not completely prevent the interactions between the antisera and the modified polynucleotide. This antisera binds to polytene chromosomes from larval glands of Drosophila melanogaster as visualized by fluorescent staining. Similar stainings were reported by Nordheim et al. (29). They have induced the synthesis of antibodies by immunization with a chemically brominated poly(dG-dC) [this polymer has the Z-form in physiological conditions, (28)]. Our results obtained with another antisera confirm the presence of Z-sequences in natural DNA. Preliminary experiments show that the staining is different after the reaction between the polytene chromosomes and N-OAc-AAF.

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