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Biochemistry

Conformation of poly(dG-dC)·poly(dG-dC) modified by the carcinogens *N*-acetoxy-*N*-acetyl-2-aminofluorene and *N*-hydroxy-*N*-2-aminofluorene

(chemical carcinogens/circular dichroism)

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ABSTRACT **Poly(dG-dC)·poly(dG-dC) was modified by reaction with *N*-acetoxy-*N*-acetyl-2-aminofluorene (N-AcO-AAF). Two samples with 6.6% and 8.5% modified bases were prepared. The modified bases are randomly distributed along the polymer chain, as deduced from competition experiments between antibodies against *N*-2-(guanosin-8-yl)-acetylaminofluorene, modified poly(dG-dC)·poly(dG-dC), and modified DNAs. Circular dichroism studies show that poly(dG-dC)·poly(dG-dC) modified by N-AcO-AAF is much more sensitive to the addition of alcohol than poly(dG-dC)·poly(dG-dC). In about 50% (vol/vol) alcohol, both polynucleotides have the same conformation, which is the Z form or a Z-like form. Moreover, in low salt and in the absence of alcohol, poly(dG-dC)·poly(dG-dC) modified by N-AcO-AAF is partially in the Z form. Poly(dG-dC)·poly(dG-dC) modified by *N*-hydroxy-*N*-2-aminofluorene can also adopt the Z form, but the transition is induced at a higher percentage than that of poly(dG-dC)·poly(dG-dC) modified by N-AcO-AAF. In low salt and in the absence of alcohol, no Z form was detected in poly(dG-dC)·poly(dG-dC) modified by *N*-hydroxy-*N*-2-aminofluorene.**

It seems to be generally accepted that most and perhaps all chemical carcinogens bind covalently to DNA. This covalent binding can distort the double helix, which might be of importance in the tumorigenic process. Numerous studies have been done with *N*-hydroxy-*N*-acetyl-2-aminofluorene. After administration of this compound to rats, at least three addition DNA products are formed. Two of them have been identified as *N*-(deoxyguanosin-8-yl)-acetylaminofluorene and as 3-(deoxyguanosin-*N*-2-yl)-acetylaminofluorene. These two products are also formed *in vitro* by the nonenzymatic reaction between DNA and *N*-acetoxy-*N*-acetyl-2-aminofluorene (N-AcO-AAF), and the major product is the C(8) derivative (for a general review, see ref. 1). Physicochemical studies of the *in vitro* modified DNA have led to the proposal of the insertion–denaturation model in which the C(8)-substituted guanines are outside the double helix and the fluorene ring is inside. This also agrees with the base displacement model deduced from the study of modified oligonucleotides (for reviews, see refs. 2 and 3).

In addition to this local distortion of DNA, one can wonder whether these modified bases could favor or hinder conformational changes of some DNA sequences. Depending upon the experimental conditions, synthetic and natural DNAs can adopt different forms—for example, the A or the B form. We have chosen to study acetylaminofluorene (AAF)-modified poly(dG-dC)·poly(dG-dC) as a model compound. The reaction between N-AcO-AAF and poly(dG-dC)·poly(dG-dC) occurs mainly on the C(8) of guanine residues (4), as found in the *in vitro* reaction with DNA. On the other hand, the conformation of poly(dG-dC)·poly(dG-dC) is sensitive to the nature of the solvent (5, 6). In this work the conformation of AAF-modified poly(dG-dC)·poly(dG-dC) has been characterized by circular dichroism. Moreover, the importance of the nature of the carcinogen was investigated by comparing the conformation of poly(dG-dC)·poly(dG-dC) modified by *N*-hydroxy-*N*-2-aminofluorene (N-OH-AF) and by N-AcO-AAF.

MATERIALS AND METHODS

Poly(dG-dC)·poly(dG-dC) was purchased from Boehringer Mannheim. The sedimentation coefficient in 0.1 M NaCl/5 mM Tris·HCl, pH 7.5, at 20°C was 5.7 S. Purification of *Micrococcus lysodeikticus* DNA (70% G+C) and calf thymus DNA (42% G+C) has been described (7).

The reaction between nucleic acids and N-AcO-AAF or N-OH-AF was performed as described (8–10) with minor modifications. Briefly, poly(dG-dC)·poly(dG-dC) (150 μg) was dissolved in 2 ml of 2 mM phosphate buffer (pH 7.2), and 10 μl of an alcoholic solution of N-AcO-AAF was added (the ratio of carcinogen to phosphate was 0.2–0.3). After incubation at 37°C for 3 hr, the solution was extracted three times with ethyl ether. The solvent was evaporated under reduced pressure. The polymer was dissolved in 200 μl of 0.1 M NaCl and then precipitated by addition of ethanol. The polymer was dissolved in 1 mM phosphate buffer and exhaustively dialyzed against the same buffer.

The reaction with N-OH-AF was performed in 10 mM citrate buffer (pH 5); N-OH-AF was dissolved in dimethyl sulfoxide. After the treatment with ether, the solution was extracted twice with phenol saturated with buffer, precipitated by addition of alcohol, and then exhaustively dialyzed.

The percentages of modified bases were deduced from the ultraviolet absorption spectra. We will write ML DNA-AAF, CT DNA-AAF, and poly(dG-dC)-AAF for *M. lysodeikticus* DNA, calf thymus DNA, and poly(dG-dC)·poly(dG-dC), respectively, after reaction with N-AcO-AAF, and poly(dG-dC)-AF for poly(dG-dC)·poly(dG-dC) after reaction with N-OH-AF.

Radioimmunoassays with antibodies to (guanosin-8-yl)-acetylaminofluorene (Guo-AAF) were performed as described (11) (the antibodies were further purified on a Sepharose-DNA-AAF column). Ultraviolet absorption and circular dichroism spectra were recorded with a Cary 15 spectrophotometer and a Roussel Jouan III dichrograph, respectively. In

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Abbreviations: N-AcO-AAF, *N*-acetoxy-*N*-acetyl-2-aminofluorene; N-OH-AF, *N*-hydroxy-*N*-2-aminofluorene; AAF, acetylaminofluorene; AF, aminofluorene; Guo-AAF, *N*-2-(guanosin-8-yl)-acetylamino-fluorene; DNA-AAF, DNA that has reacted with N-AcO-AAF; poly(dG-dC)-AAF, poly(dG-dC)·poly(dG-dC) that has reacted with N-AcO-AAF; poly(dG-dC)-AF, poly(dG-dC)·poly(dG-dC) that has reacted with N-OH-AF.

the experiments as a function of alcohol concentration, increasing volumes of ethanol were added to the polynucleotide solution; the percentage was expressed as the ratio of added volume of alcohol to the sum of the buffer and ethanol volumes.

RESULTS

Radioimmunoassays. In the reaction between poly(dG-dC)·poly(dG-dC) and N-AcO-AAF, substitution occurs mainly on the C(8) of guanine residues (4). The affinities of antibodies against Guo-AAF to native DNA-AAF, denatured DNA-AAF, and poly(dG-dC)-AAF were compared to determine whether the modified guanine residues in poly(dG-dC)-AAF were randomly distributed along the polynucleotide chain. Competitive radioimmunoassays between the tracer [^{3}H]Guo-AAF, the highly purified antibodies against Guo-AAF, and several ligands were performed. In Fig. 1, the inhibition of the tracer binding to the antibodies is plotted as a function of the logarithm of the inhibitor concentration. Guo-AAF inhibited better than the modified DNA, which agrees with previous results (11). On the other hand, the three modified DNAs inhibited the tracer–antibody binding equally efficiently.

Ultraviolet Spectra. Even at low ionic strength, poly(dG-dC)-AAF and poly(dG-dC)-AF are multistranded helices. The variation of the absorbance as a function of temperature at 310 nm (at which only the fluorene ring absorbs) and at 275 nm were those expected for cooperative meltings (results not shown).

Circular Dichroism. The circular dichroism spectra of poly(dG-dC)·poly(dG-dC) in 1 mM phosphate buffer (pH 7.2) and in alcohol/phosphate buffer mixture (60% by vol alcohol) are shown in Fig. 2. In agreement with previous results (6), the circular dichroism of poly(dG-dC)·poly(dG-dC) in the presence of ethanol was nearly an inversion of that in low salt concentration.

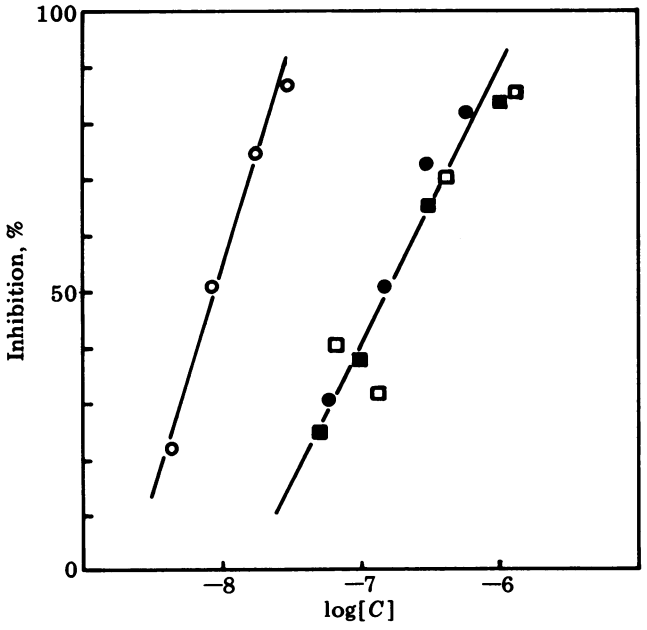

FIG. 1. Radioimmunoassays. Inhibition of [^{3}H]Guo-AAF binding as a function of the logarithm of the inhibitor concentration. Inhibitor: ○, Guo-AAF; ●, denatured CT DNA-AAF (7.8% modified bases); ■, native ML DNA-AAF (7% modified bases); □, poly(dG-dC)-AAF (6.6% modified bases). Antibody concentration, 15 nM; [^{3}H]Guo-AAF, 15 nM. The concentrations of modified DNAs are expressed in mol of bound carcinogens. The solvent was 0.2 M NaCl/5 mM Tris·HCl, pH 7.5/0.1 mM EDTA; the temperature was 4°C.

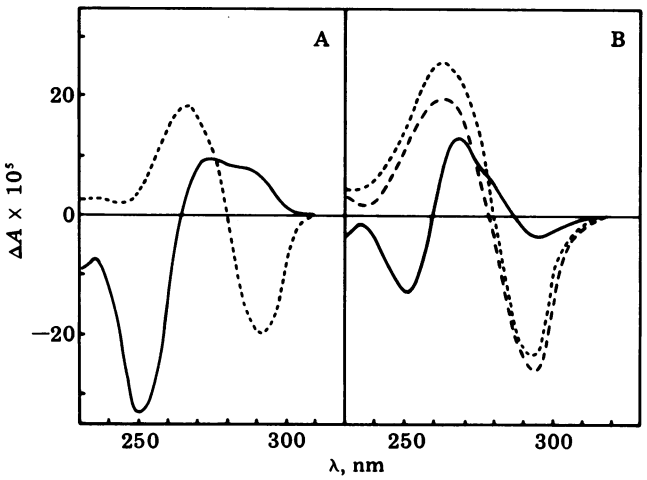

FIG. 2. Circular dichroism spectra. (*A*) Poly(dG-dC)·poly(dG-dC) in 1 mM phosphate buffer (pH 7.2) (—) and 60% ethanol (- - -). (*B*) Poly(dG-dC)-AAF (8.5% modified bases) in 1 mM phosphate buffer (pH 7.2) (—); 40% ethanol (- - -); and 4 M $NaClO_4$/5 mM phosphate buffer, pH 7.2 (– –). $\Delta A = \Delta A_L - A_R$ is the difference in absorption between left and right polarized light at wavelength λ. The absorbance at 260 nm was 0.4; temperature was 25°C.

The circular dichroism spectrum of poly(dG-dC)-AAF in 1 mM phosphate buffer had a negative band centered at 295 nm, a positive band at 270 nm, and a negative band centered at 250 nm. The relative intensities of the bands depended upon the percentage of bound AAF residues (Figs. 2 and 3). The spectrum was markedly modified by addition of ethanol. The first negative band became more intense whereas the negative band centered at 250 nm decreased and then became positive with a red shift of the maximum (Fig. 3). The circular dichroism spectra of poly(dG-dC)-AAF in 40% alcohol or in 4 M $NaClO_4$ were similar to those already observed for poly(dG-dC)·poly(dG-dC). The transitions of poly(dG-dC)·poly(dG-dC) and of poly(dG-dC)-AAF were cooperative, as shown, for example, by the variation of $\Delta A_{290\ nm}$ as a function of the percentage of alcohol (Fig. 3).

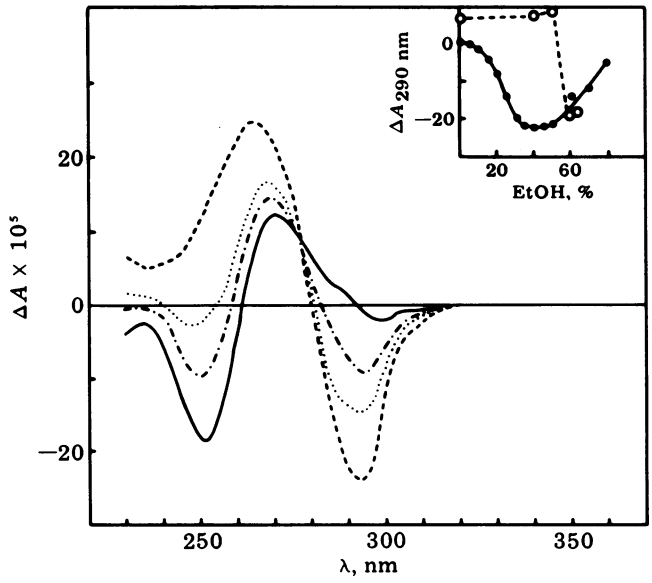

FIG. 3. Circular dichroism spectra. Poly(dG-dC)-AAF (6.6% modified bases) in 1 mM phosphate buffer (—); 20% ethanol (- - -); 25% ethanol (·····); and 40% ethanol (– –). (*Inset*) Variation of $\Delta A_{290\ nm}$ as a function of the percentage of ethanol. ●, Poly(dG-dC)-AAF (6.6% modified bases); ○, poly(dG-dC)·poly(dG-dC). The absorbance at 260 nm was 0.4; temperature was 25°C.

In Fig. 4 are shown the difference spectra between the spectrum of poly(dG-dC)·poly(dG-dC) in 60% alcohol and the spectrum of poly(dG-dC)·poly(dG-dC) in 1 mM phosphate buffer and between the spectra of poly(dG-dC)-AAF (6.6% modified bases) or poly(dG-dC)-AAF (8.5% modified bases) and the spectrum of poly(dG-dC)·poly(dG-dC) in 1 mM phosphate buffer. The shapes of the difference spectra were similar, with a minimum at 290 nm and a maximum at 253 nm. There were slight differences above 305 nm which reflect the contribution of AAF residues.

We have also studied the effect of alcohol on the conformation of poly(dG-dC)-AF (6% modified bases). In 1 mM phosphate buffer, the circular dichroism spectrum had a weak positive band due to the aminofluorene (AF) residues [the circular dichroism spectrum of native DNA-AF (6% modified bases) has a negative band above 300 nm; ref. 10]. Below 300 nm, the spectrum was similar to that of poly(dG-dC)·poly(dG-dC). Addition of alcohol (Fig. 5) resulted in modifications of the spectra. The transition was also cooperative, but occurred at a higher percentage in contrast to poly(dG-dC)-AAF.

DISCUSSION

In this work, we have studied the conformation of poly(dG-dC)·poly(dG-dC) modified by two chemical carcinogens and of poly(dG-dC)·poly(dG-dC) under various experimental conditions. We show that poly(dG-dC)-AAF is much more sensitive to the addition of alcohol than poly(dG-dC)·poly(dG-dC) or poly(dG-dC)-AF. The covalently bound AAF residues favor a conformational transition of poly(dG-dC)·poly(dG-dC).

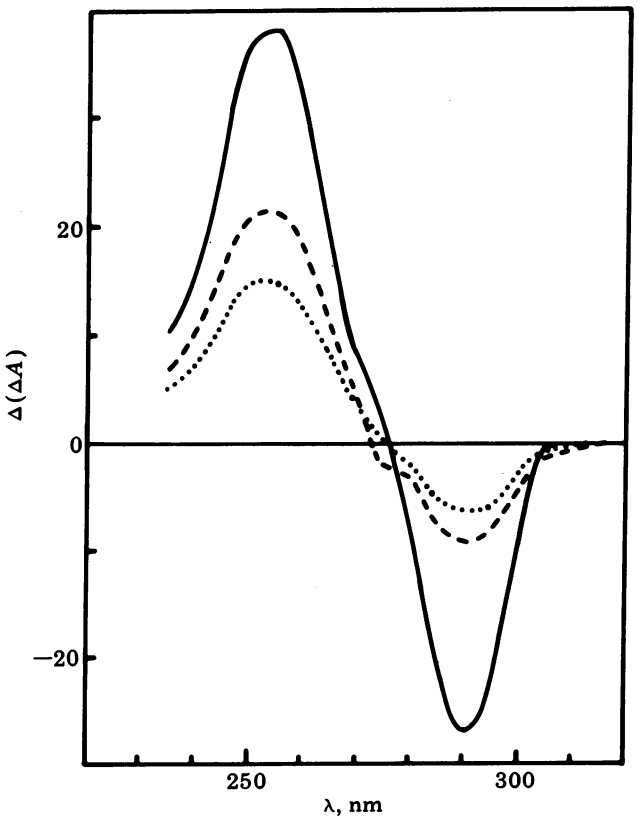

FIG. 4. Difference circular dichroism spectra. Difference spectra between poly(dG-dC)·poly(dG-dC) in 60% ethanol and poly(dG-dC)·poly(dG-dC) in 1 mM phosphate buffer (—), between poly(dG-dC)-AAF (6.6% modified bases) and poly(dG-dC)·poly(dG-dC) in 1 mM phosphate buffer (·····), and between poly(dG-dC)-AAF (8.5% modified bases) and poly(dG-dC)·poly(dG-dC) in 1 mM phosphate buffer (– –). The differences were calculated from the spectra shown in Figs. 2 and 3.

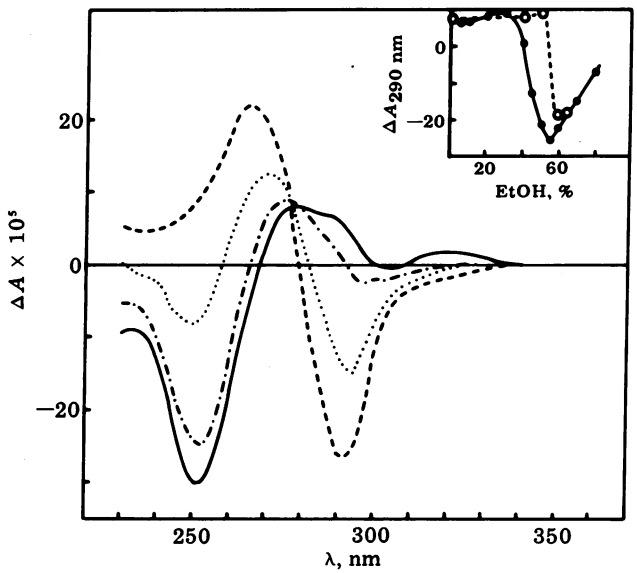

FIG. 5. Circular dichroism spectra. Poly(dG-dC)-AF (6% modified bases) in 1 mM phosphate buffer (—); 40% ethanol (– · –); 45% ethanol (·····); and 55% ethanol (– – –). (*Inset*) Variation of $\Delta A_{290\ nm}$ as a function of the percentage of alcohol. ●, Poly(dG-dC)-AF (6% modified bases); ○, poly(dG-dC)·poly(dG-dC). The ultraviolet absorbance was 0.4 at 260 nm.

In poly(dG-dC)-AAF (6.6% modified bases), there is no strong clustering of the substituted bases. This is deduced from competition experiments between the antibodies against Guo-AAF, [^{3}H]-Guo-AAF, and DNA-AAF (native or denatured) or poly(dG-dC)-AAF. The three modified DNAs inhibit tracer–antibody binding equally efficiently. After reaction between DNA and N-AcO-AAF, there is no cluster of dGuo-AAF residues. This can be verified directly by the electron microscopic visualization of the N-AcO-AAF binding sites in DNA (less than 1% of the bases were modified) by means of the antibodies to Guo-AAF (12). Also, in the precipitate formed by DNA-AAF (5.7% modified bases) and anti-DNA-AAF specific IgG molecules, the molar ratio was about two AAF residues per IgG molecule (13). If there were clusters of AAF residues in this highly modified DNA, the ratio would have been larger than 2 [one can estimate that each antibody binding site covers at least three to four base pairs (14, 15)]. A ratio larger than 2 was found in the precipitate between the antibodies against DNA-AAF and DNA-AAF (23% modified bases) (13). In this work, we found that DNA-AAF and poly(dG-dC)-AAF bind to the antibodies against Guo-AAF with the same affinity. Because there are no AAF residue clusters in DNA-AAF, we assume a statistical distribution of AAF residues in poly(dG-dC)-AAF.

Pohl *et al.* (16) provided evidence that at low salt concentration the conformation of poly(dG-dC)·poly(dG-dC) was that of B-DNA. They have also shown that raising the salt concentration or adding alcohol produced a cooperative transition from the B form to another form (5, 6). On the other hand, Wang *et al.* (17) have proposed a new structure termed Z-DNA from the studies of the structure of the hexanucleotide $(dG\text{-}dC)_3$. This fragment crystallizes as a left-handed double-helical molecule with Watson–Crick base pairs. The cytidine residues have the *anti* conformation whereas the guanosine residues have the *syn* conformation. According to Wang *et al.*, the high-salt form of poly(dG-dC)·poly(dG-dC) is the Z form, which agrees with a nuclear magnetic resonance study of

$(dG\text{-}dC)_8$ (18) and an x-ray study of poly(dG-dC)·poly(dG-dC) (19).

The conformation of poly(dG-dC)-AAF was studied by circular dichroism. We emphasize that the circular dichroism of the high-salt form or 60% alcohol form (Z form or Z-like form) of poly(dG-dC)·poly(dG-dC) is nearly an inversion of the low-salt form (B form) and that the bands are intense and have a well-defined shape (refs. 5 and 6 and Fig. 1). Circular dichroism is a convenient and efficient method for detection of the presence of any of these two forms.

The circular dichroism spectrum of poly(dG-dC)-AAF is very sensitive to the addition of ethanol, and the changes in the spectra are similar to those observed with poly(dG-dC)·poly(dG-dC). In poly(dG-dC)·poly(dG-dC), $\Delta A_{290\ nm}$ is positive and becomes negative as the percentage of alcohol is increased. In poly(dG-dC)-AAF, $\Delta A_{290\ nm}$ is null or negative and becomes more and more negative. In both cases, $\Delta A_{250\ nm}$ is negative and becomes less and less negative and then positive. The transitions of poly(dG-dC)·poly(dG-dC) and of poly(dG-dC)-AAF are cooperative, but much less alcohol is necessary to induce the transition of poly(dG-dC)-AAF in contrast to poly(dG-dC)·poly(dG-dC). We do not know the effect of alcohol on the optical activity of the AAF residues. It seems to us unlikely that the observed effects on poly(dG-dC)-AAF are only due to a solvent effect on the activity of the AAF residues because almost the same circular dichroism spectra are observed in 40% alcohol and in 4 M $NaClO_4$. Under these experimental conditions, the circular dichroism of poly(dG-dC)-AAF is similar to that of poly(dG-dC)·poly(dG-dC) in the Z form. Thus we conclude that the covalent binding of AAF residues to the C(8) of guanine residues favors the transition of the modified poly(dG-dC)·poly(dG-dC) from the B form to the Z form or to a Z-like form.

An interesting result is that even in 1 mM phosphate buffer, in the absence of alcohol, some residues of poly(dG-dC)-AAF adopt the Z form, as clearly shown by the difference circular dichroism spectra. The shapes of these difference spectra are similar to the shape of the (Z form − B form) spectrum in the range 300–240 nm, but the intensities of the bands are smaller. Thus, the Z form can be induced by the covalent binding of AAF residues but we do not know how many residues are required and how close they have to be. The percentage of Z form in the two poly(dG-dC)-AAF samples can be calculated. By linear extrapolation, one finds that a sample of poly(dG-dC)-AAF having 25% substituted bases is completely in the Z form. This last point has to be confirmed by a more complete titration curve.

Studies of modified oligonucleotides have shown that the AAF-guanosine residues are forced into the *syn* conformation (20–23). In natural DNA, once fluorene and guanine residues are covalently bound, there are at least two possibilities. If the base sequence around a modified guanine maintains the B form, the fluorene ring will be inserted into the double helix and the guanine will be outside [insertion–denaturation model (2, 9, 24, 25) or base displacement model (3, 20, 26) in which the cytidine residues are no longer paired with modified guanine residues]. Nevertheless, this is a favorable event for inducing the Z form because the modified guanine already has the *syn* conformation. If the modification occurs in a G+C-rich sequence, a dinucleotide unit or a sequence may adopt the Z form. In the Z form, the modified and unmodified guanine residues have the *syn* conformation and are paired with cytosine residues; thus the fluorene residues are outside the double helix. As noted by Wang *et al.* (16) in Z-DNA segments, the accessibility of the C(8) position is better than in B-DNA segments. In the Z form, poly(dG-dC)-AAF is expected to be stable because there is no defect in the double helix. We found that $\Delta A_{290\ nm}$ of poly(dG-dC)-AAF was constant between 30% and 55% alcohol.

In the *in vitro* reaction between N-OH-AF and DNA, there are several lines of evidence that the substitution occurs mainly on the C(8) of guanines (1, 10, 27). We assume a similar reaction with poly(dG-dC)·poly(dG-dC). At low salt concentration, the circular dichroism spectrum of poly(dG-dC)-AF has a weak positive band centered at about 320 nm, as for the monomer Guo-AF (28). Below 300 nm, the spectrum is similar to that of poly(dG-dC)·poly(dG-dC). The cooperative transition (B form → Z form) can be induced by the addition of alcohol, but more alcohol is needed than for poly(dG-dC)-AAF. The variation of $\Delta A_{290\ nm}$ of poly(dG-dC)-AF as a function of the percentage of alcohol is similar to that of poly(dG-dC)·poly(dG-dC) in the range 0–30%. The simplest explanation of these results is that the AF residues do less to force the modified guanines into the *syn* conformation than the AAF residues and, consequently, chemical substitution of guanines by AF residues is much less efficient at inducing the transition to the Z form than substitution by AAF residues. This agrees with a nuclear magnetic resonance study of Guo-AAF and Guo-AF. Striking differences appear in the resonances of the ribose protons which strongly suggest a *syn* conformation for Guo-AAF and an *anti* conformation for Guo-AF.

Finally, in 1 mM phosphate buffer, in the absence of alcohol (or even in the presence of 30% alcohol), the circular dichroism spectra are almost identical to that of poly(dG-dC)·poly(dG-dC) in 1 mM phosphate buffer. Under these experimental conditions, no Z form in poly(dG-dC)-AF was detected.

In conclusion, one can say that if guanine residues in DNA bind covalently to AAF residues, depending upon the respective positions of the modified guanines, the base sequences, and the local conditions, two effects can be expected: a distortion of the double helix with the modified guanine residues outside the helix and the fluorene residues inside (insertion–denaturation model) or a sequence conformational change (Z form or Z-like form) with the modified guanine residues inside the helix, paired with cytosine residues, and the fluorene ring outside the helix. This might be important for cellular events that follow the chemical modification of DNA and, more especially, for the behavior of the DNA replication machinery and DNA repair enzymes.

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