Strong structural effect of the position of a single acetylaminofluorene adduct within a mutation hot spot

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ABSTRACT

The *NarI* restriction enzyme recognition site, $G_1G_2CG_3CC$, has been identified as a hotspot for −2 frameshift mutations induced by N-2-acetylaminofluorene (AAF) on the basis of a forward mutation assay in plasmid pBR322 in the bacterium *Escherichia coli* . AAF binds primarily to the C-8 position of guanine residues, and the three guanines of the *NarI* site are similarly reactive. Despite this similar chemical reactivity, only binding of AAF to the G_3 residue causes the −2 frameshift mutations. To study the mechanisms underlying the specificity of the mutagenic processing further, we monitored the structural changes induced by a single AAF adduct within the *NarI* site by means of CD spectroscopy and thermal denaturation.The *NarI* sequence was studied as part of the 12-mer ACCGGCGCCACA. The purification and characterization of the three isomers having a single AAF adduct covalently bound to one of the three guanines of this 12 mer are described. The analysis of the melting profiles of the duplexes formed when these three isomers are annealed with the oligonucleotide of complementary sequence shows the same destabilizing effect of the AAF adduct on the three DNA helices. It is also shown, from the CD spectra, that modification of guanine G_1 or G_2 by AAF does not induce major changes in the helical structure of DNA. On the other hand, modification of guanine G_3 induces a change in the CD signal that suggests the formation of a local left handed structure within the 12-mer duplex. These results show the polymorphic nature of the DNA structure in the vicinity of an AAF adduct.

INTRODUCTION

To understand the effect of covalent binding of chemical agents on DNA, it is essential to investigate the relationship between the change in conformation undergone by the DNA and the biological fate of the resulting adducts.

N-Acetoxy-N-2-acetylaminofluorene (N-AcO-AAF), a model for the strong rat liver carcinogen N-2-acetylaminofluorene (AAF) has been shown, by means of a forward mutation assay based on the inactivation of the tetracycline-resistance gene of pBR322, to induce mainly frameshift mutations (more than 90%) (1, 2). These mutations occur within two types of sequences : i) runs of guanines and ii) the sequence GGCGCC, recognized by the restriction enzyme *NarI* . The mutations in runs of guanines are primarily −1 frameshift mutations that can be related to the slippage mechanism described by Streisinger and coworkers (3, 4). The −2 frameshift mutations occurring at the *NarI* site do not seem to result from the same mechanism, in that the two pathways have different genetic requirements (2, 5). The comparison between the mutation spectrum and the chemical binding spectrum for AAF suggested that the high mutation frequency observed within the *NarI* sequence results from the processing of an unusual DNA conformation induced by the AAF adduct (6). The importance of the structure became even more obvious when it was shown by means of single-adduct mutagenesis experiments that only binding

of AAF to the third guanine (G_3) in the *NarI* site $G_1G_2CG_3CC$ is responsible for the mutagenic event (7).

To get further insight into the mechanisms of this strong specificity, a 12 mer containing the *NarI* site, $^{5'}$d-ACCGGCGCCACA $^{3'}$ was synthesized and reacted *in vitro* by N-AcO-AAF. This paper presents the purification of each isomer of position of the AAF adduct recovered from the reaction mixture of N-AcO-AAF with the 12-mer, the structural features revealed by circular dichroïsm (CD) spectroscopy and the melting profiles of the helices formed when these isomers are hybridized with the 12-mer of complementary sequence.

MATERIAL AND METHODS

a) Enzymatic reactions

All enzymes were purchased from New England Biolabs, Beverly, MA. Reactions were performed under the conditions specified by the manufacturer.

b) Preparation of AAF-monomodified 12 mers.

The 12-mer $^{5'}$d (ACCGGCGCCACA) $^{3'}$ was synthesized on an automated Applied Biosystem instrument, using phosphoramidite chemistry. The full length deprotected oligonucleotides were purified by means of reversed phase high performance liquid chromatography (HPLC), as previously described (8). The reactions between nucleic acids and N-AcO-AAF were performed as described (8) , with minor modifications : the 12 mer was resuspended at a concentration of 0.5 mg/ml in 2 mM citrate buffer, pH 7.2, and preincubated at 37°C. A 4-fold molar excess of N-AcO-AAF, dissolved in ethanol, was added so that the final solution contained 5% ethanol. The reaction mixture was incubated at 37°C for 6 min, and the reaction was stopped by removing unbound fluorene derivatives by 3 consecutive chloroform extractions.

Complete purification of the three AAF monoadducted isomers required two successive applications of reversed phase HPLC on a C18 Nucleosil column, purchased from Bischoff, Leonberg, Germany. The first solvent program was an isochratic step at 24% methanol in tri ethylamine acetate (TEAA), 0.05 M, pH6 for 10 mins, followed by a linear gradient to 43% methanol in 50 min (method A). The solvent program for the second purification also started with a 10 min isochratic step at 15% of a mix methanol-acetonitrile (1:1 in volume) in TEAA, 0.05M, $MgCl_2$, 1mM, pH 7.2, followed by a linear gradient to 27% of the mix methanol-acetonitrile in 50 min (method B)

The chromatography fractions containing the AAF-monoadducted isomers were lyophilized and ethanol-acetate precipitated.

c) Characterization of the monoadducted oligonucleotides

The analysis of the position of the guanine bearing the AAF adduct was performed with the T4 DNA polymerase assay (6). This assay is based on the observation that the 3'->5' exonuclease activity of the T4 DNA polymerase is stopped one nucleotide prior to the AAF adduct. 50 ng of unmodified and AAF monomodified 12 mer were 5' phosphorylated in a final volume of 10 μl by incubation with 2u of T4 polynucleotide kinase and a 4-fold molar excess of ATP for 30 min at 37°C in the presence of kinase buffer. γ-^{32}P ATP (3000 Ci/mM) was diluted with unlabeled ATP to reach a final specific activity of 1000 Ci/mmol.

2 ng (1 μl) of each phosphorylated oligonucleotide were mixed with 4 ng of the complementary oligonucleotide in 100 μl of T4 DNA polymerase buffer. The mixtures were incubated for 2 min at 95°C, then hybridization was allowed to proceed until the solution was at room temperature. 0.1 u of T4 DNA polymerase was added to 20 μl of

each of the duplex solutions, and digestion was carried out for 10 min at 37°C. Each DNA was ethanol-acetate precipitated in the presence of carrier tRNA, dissolved in loading buffer, and subjected to electrophoresis on a 20% sequencing gel.

d) CD and UV spectroscopy

All CD and UV measurements, unless noted, were performed in a 10 mM Tris buffer containing 50 mM NaCl, 1mM ethylene diamine tetraacetic acid (EDTA). The DNA concentration was, for all spectra, 40 μg/ml.

Circular dichroism spectra were recorded using a Jobin Yvon spectrometer DC III. The ellipticity was measured at ambient temperature in quartz cylindrical cuvettes with an optical path length of 1 cm.

RESULTS

a) Purification of the three monoadducted 12-mer oligonucleotides

N-AcO-AAF mainly reacts with the C(8) of guanine residues in single stranded DNA (9,10). Thus, the crude reaction mixture resulting from the modification of the 12-mer $^{5'}$d(ACCG$_1$G$_2$CG$_3$CCACA)$^{3'}$ with N-AcO-AAF contains a mixture of 8 species : the non modified-, 3 possible monoadducted-, 3 possible diadducted- and one triadducted-oligonucleotides. The HPLC profile of this mixture under conditions A (see Materials and Methods) is presented in figure 1A. The presence of AAF adducts in the oligonucleotides was characterized by UV spectroscopy. A red shift of the maximum at 257 nm and a shoulder at 305 nm are observed (11). Ratios of optical densities at 305 nm and 260 nm of .12, .23 and .26 identify the presence of 1, 2 and 3 -AAF adducts, respectively. Peaks (a) and (b) (figure 1) were thus characterized as containing monoadducted 12-mer oligonucleotides. The quantitative analysis of our UV spectra (not shown) confirmed that our experimental conditions were optimum for the production of monoadducted molecules (40%), according to Poisson statistics. The products collected in peaks (a) and (b) were analysed, using the T4 polymerase assay (6). Peak (a) was identified as containing pure 12-mer monoadducted on guanine G$_1$, which we refer to as Nar-AAF1, and peak (b) contains a mixture of 12-mer reacted at guanine G$_2$ (Nar-AAF2) or G$_3$ (Nar-AAF3) respectively (Figure 2, panel B, lane a and b). Peak (b) was further purified under conditions B, as shown in Figure 1B. The position of the adduct and the purity of the products collected in each peak was established by the T4 DNA polymerase assay. Finally the purity of each monoadducted oligonucleotide was judged to be $\geq$ 95% from the analysis shown in Figure 2, lanes a, c and d.

b) AAF destabilizes the NarI duplex

The non-modified 12-mer and each monoadducted 12-mer were hybridized with a 12 base long oligonucleotide of complementary sequence : the corresponding duplexes are referred to as Nar, Nar-AAF1, Nar-AAF2, and Nar-AAF3, according to the adducted strand. The variations of the absorbance at 260nm versus temperature clearly indicate a cooperative melting of the 4 helices (Figure 3). The melting temperature and the hyperchromicity (defined as the ratio (OD_{260}(final)$-$$OD_{260}$(initial))/$OD_{260}$(initial) where OD_{260} is the optical density at 260 nm) of the duplexes are summarized in table 1. All three monoadducted duplexes are destabilized to the same extent (decrease of $\approx 10-13$°C of the melting temperature) compared to the non modified Nar duplex. There is also a 20% decrease in the hyperchromicity for each monoadducted duplex. Qualitatively similar results have been reported for calf thymus DNA randomly modified with N-AcO-AAF (15)

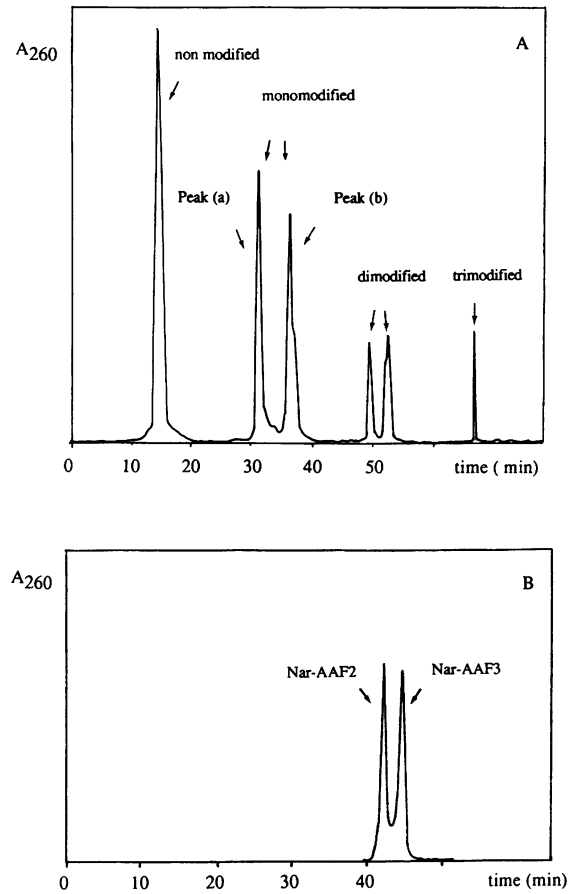

Figure 1. Panel A shows a HPLC profile of the crude mixture resulting from the reaction of N-Aco-AAF with the 12 mer d(ACCGGCGCCACA). Peak (b), containing the oligonucleotides monoadducted on guanine G2 or G3, was repurified by HPLC, as presented in panel B. The gradient conditions are given under Material and Methods.

c) CD spectra suggest that an unusual DNA conformation is induced within Nar-AAF3

Figure 4 shows that each position of the AAF within the NarI sequence has a different effect on the CD spectra recorded at low ionic strength (50 mM NaCl, each sample at DNA concentration of 40 μg / ml).

The spectrum of the Nar duplex is similar to the spectra observed for DNA of high G+C content in B form (12, 13, 14), with a marked positive band around 290nm.

The circular dichroïsm induced in the -AAF adduct prevents a simple interpretation of the CD spectra of the monoadducted duplexes. However, changes in the structure of DNA can be observed in the 230−260 nm region, based on the observation that the absorption spectra of AAF is minimal within this region and maximal in the 270−290nm region (16).

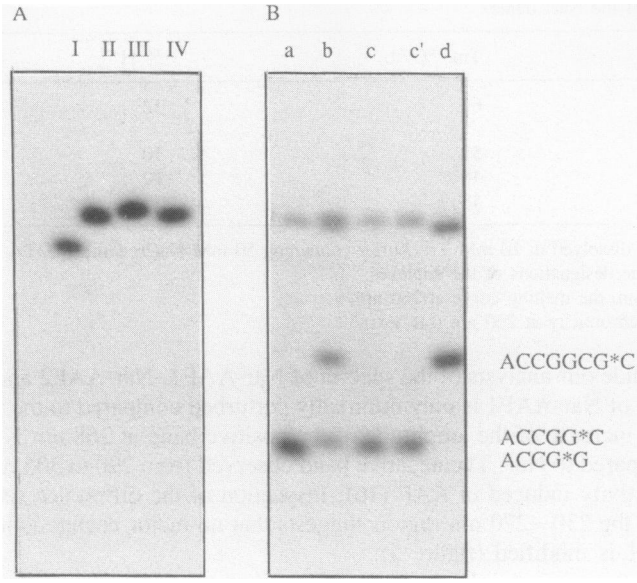

Figure 2. T4 DNA polymerase analysis of the purified AAF monoadducted isomers of the 12 mer d(ACCGGCGCCACA). All oligonucleotides are [^{32}P] 5′ end labeled. The analysis is performed on a 20% polyacrylamide gel. Panel A : lane I shows the non modified 12 mer. The AAF adduct is responsible for a slightly slower migration of the monoadducted 12 mers (lanes II, III, IV). Panel B : the 3′ → 5′ exonuclease activity is blocked one nucleotide before the AAF adduct, hence revealing the position of the modified guanine in the 12 mer (6). Digestion of the product collected in peaks (a) and (b) of the HPLC chromatography profile in figure 1a indicates that (a) contains pure Nar-AAF1 (lane a) and (b) contains a mixture of Nar-AAF2 and Nar-AAF3 (lane b). The second purificaton of peak b by method B (see Materials and Methods) resolved Nar-AAF2 and Nar-AAF3, as confirmed by the digestion of the products collected in the two corresponding peaks (figure 1, panel B) by T4 DNA polymerase (lane c and d). Lane c′ is a duplication of lane c.

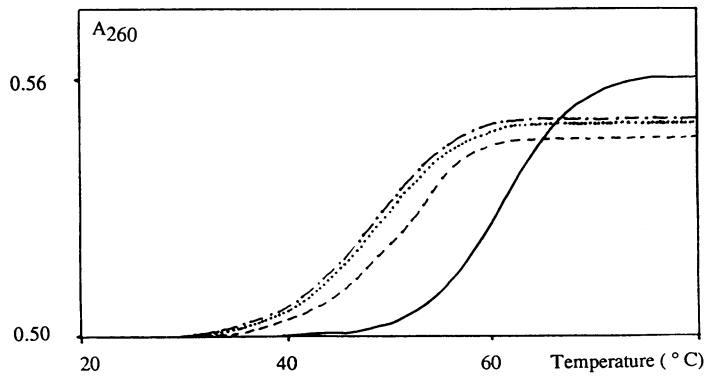

Figure 3. Stability of the AAF monoadducted duplexes. Melting curves at 260 nm of the 12 mer Nar duplex d(ACCGGCGCCACA) (——), Nar-AAF1 duplex (- - -), Nar-AAF2 duplex (· · ·) and Nar-AAF3 duplex (— · — ·). Each DNA was dissolved in 10 mM Tris buffer containing 50 mM NaCl and 1 mM EDTA. The curves were normalized to 0.5 OD at 20°C.

Table I: Stability of the NarI duplex

Duplex [a]	Tm, °C [b]	% H [c]
Nar	61	12
Nar-AAF1	51	10
Nar-AAF2	48	10
Nar-AAF3	48	10

[a] Each duplex was dissolved in 10 mM Tris buffer containing 50 mM NaCl, 1 mM EDTA. See the text for an explanation of the designations of the duplexes.
[b] Tm is defined from the melting curve at 260 nm.
[c] % H is the hyperchromicity at 260 nm (see text).

This fact will guide our analysis of the spectra of Nar-AAF1, Nar-AAF2 and Nar-AAF3.

The spectrum of Nar-AAF1 is only minimally perturbed compared to the non-modified duplex. A 50% increase of the amplitude of the positive band at 268 nm is observed for Nar-AAF1 compared to Nar. The negative band observed from 290 to 305 nm is assigned to the optical activity induced in AAF (16). Inspection of the difference spectrum (Nar-AAF1 − Nar) in the 230 − 270 nm region suggests that no major change is induced in the duplex when G1 is modified (figure 5).

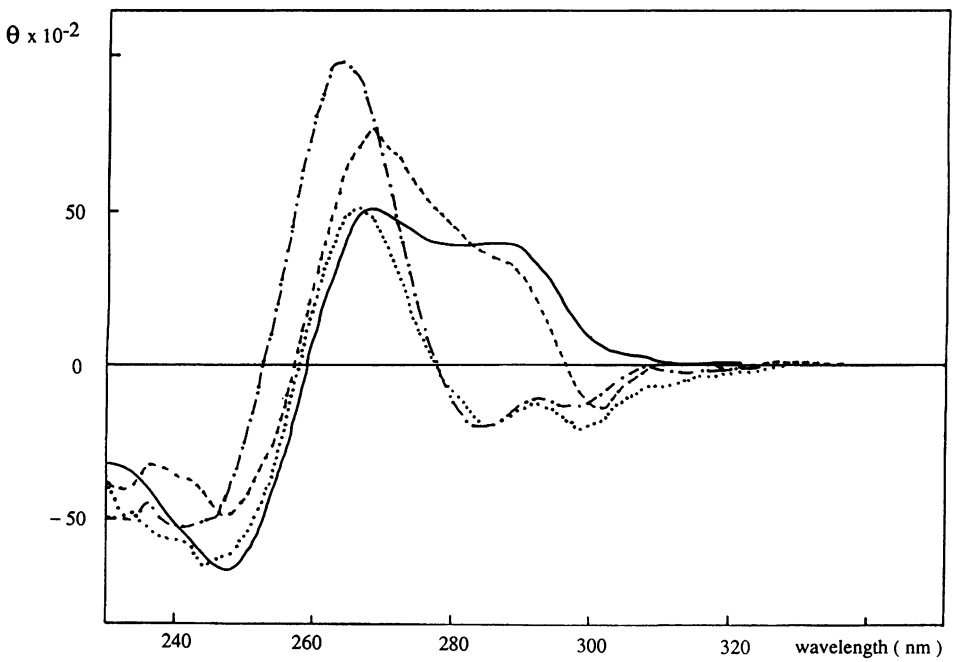

Figure 4. CD spectra of AAF monoadducted duplexes in the low ionic strength buffer TEN [10 mM Tris, 1 mM EDTA 50 mM NaCl (pH 7)] : ——— d(ACCGGCGCCACA) duplex, - - - - - Nar-AAF1 duplex, · · · · · Nar-AAF2 duplex, — · — · Nar-AAF3 duplex.

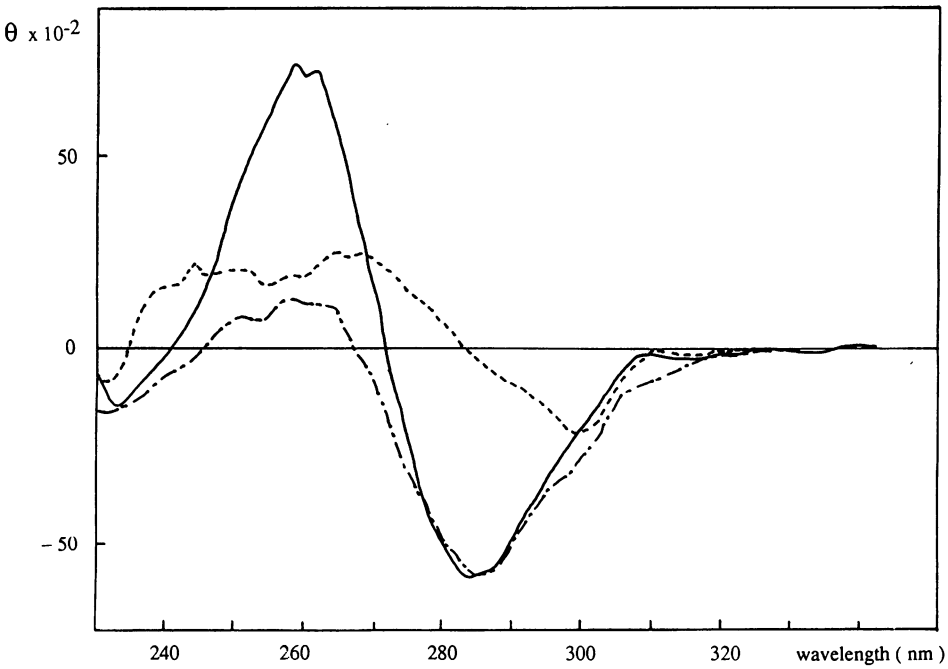

Figure 5. Difference spectra between Nar-AAF1 and the non-modified Nar duplex (- - -), between Nar-AAF2 and Nar (— · — ·), and between Nar-AAF3 and Nar (——). The differences were calculated from the spectra shown in figure 4.

The spectrum of Nar-AAF2 presents the same trough centered at 300 nm as observed for Nar-AAF1. The maximum intensity at 268 nm is not changed, but the band undergoes a slight blue shift of 2nm. A substantial change is observed in the 270−290 nm region (Figure 4 and Figure 5). The positive band centered at 280nm for the Nar duplex becomes negative for Nar-AAF2. The difference spectrum (Nar-AAF2−Nar) (see figure 5) emphasizes the presence of the negative band around 280 nm which can be attributed to the circular dichroïsm induced in the AAF moiety. No substantial difference is observed below 270 nm, indicating that no major change is induced in the overall helical structure of the oligonucleotide.

The spectrum of Nar-AAF3 is the most markedly modified compared to the unmodified duplex. The positive band undergoes an blue shift to 263 nm, and its amplitude is increased by 100%. The positive band at 280 nm observed for the unmodified duplex Nar becomes negative for Nar-AAF3 as for Nar-AAF2.

d) Addition of salt does not induce any cooperative transition of the NarI helices.

The CD spectra for Nar, Nar-AAF1, Nar-AAF2, and Nar-AAF3 duplexes at 3M NaCl are compared in figure 6. No major change in the spectra is seen for any of the four samples at high salt concentration relative to the spectrum at low salt concentration. The differences in CD spectra among the different helices as observed at low ionic strength are conserved. Similar results were obtained when NaCl was replaced by $MgCl_2$, in the range 0 mM to 20 mM (results not shown).

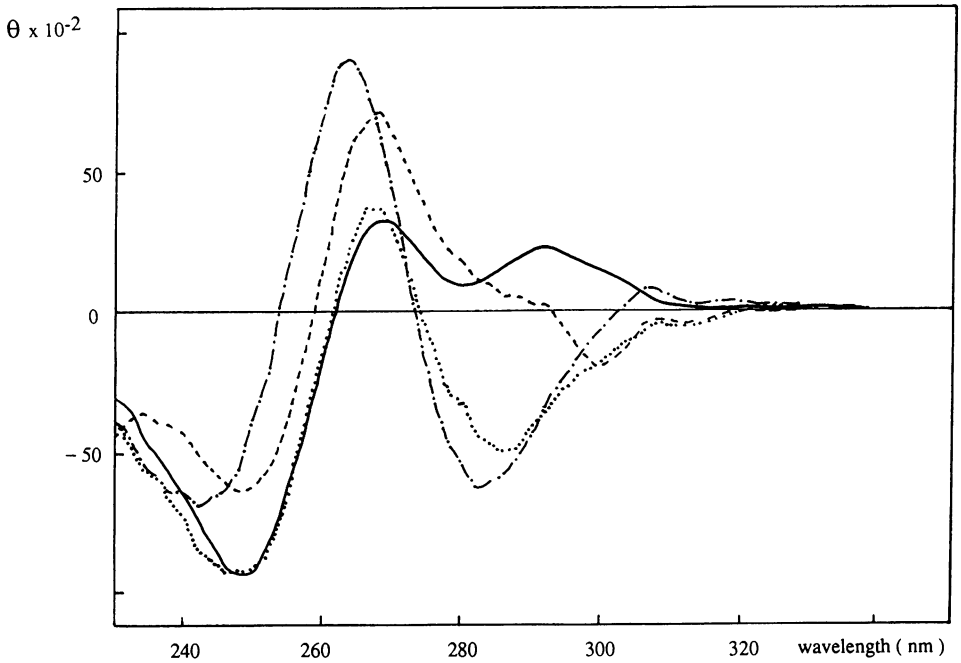

Figure 6. CD spectra of AAF monoadducted duplexes in the high ionic strength buffer TEN3M [1 mM EDTA 3 M NaCl (pH 7)] : ——— d(ACCGGCGCCACA) duplex in 10 mM Tris, - - - - - Nar-AAF1, · · · · · Nar-AAF2, — · — · Nar-AAF3.

DISCUSSION

The *NarI* sequence, $^{5'}$ $G_1G_2CG_3CC$ $^{3'}$ is a hot spot for AAF induced −2 frameshift mutagenesis (1, 2). There is a strong effect of the position of the AAF adduct within this sequence, in that only binding of AAF to the third guanine is responsible for the mutagenic event (7).

In this paper, the effect of the position of the AAF adduct on the structure of the *NarI* sequence was studied within the context of the 12-mer oligonucleotide, ACCGGCGCCACA.

N-AcO-AAF binds specifically to the C(8) position of guanine residues in single stranded DNA (9, 10). Under the normal conditions of deprotection used in the phosphoramidite method for the synthesis of oligonucleotides, the guanine-AAF residue is not stable. Therefore, it was not possible to synthezise directly the monoadducted oligonucleotides. Consequently, we prepared the modified oligonucleotides by reacting the deprotected oligonucleotide ACCGGCGCCACA with N-AcO-AAF. Among the different products that are formed during the reaction, extensive HPLC chromatography on a reverse phase column allowed us to purify the three guanine-AAF monoadducted position isomers. Though similar to the purification steps established for single adduct mutagenesis experiments (8), a new strategy had to be developed to increase the efficiency of the procedure. This was greatly helped by the finding that $MgCl_2$ can enhance separation in reverse phase liquid

chromatography. It should be noted however, that while $MgCl_2$ enabled us to separate Nar-AAF2 from Nar-AAF3 (single stranded) it was of no help when used on the crude reaction mixture.

Each monoadducted 12-mer and the non-modified 12-mer used as a control were annealed to the complementary strand. CD spectra and thermal stability of the different duplexes were recorded and analysed.

Upon random modification with N-AcO-AAF, a linear decrease of the melting temperature of high molecular weight DNA of about 1.2°C per 1% of modified bases was observed (15). Since one AAF adduct for a double stranded 12-mer corresponds to 4% of the bases modified, one would expect 5°C of destabilisation for each monoadducted Nar duplex compared to the non-modified 12-mer duplex. In fact, a destabilisation of 10°C is found experimentally. The additional destabilisation that is observed in the present work can be assigned to the instability introduced at the ends of these short duplexes.

The interpretation of CD spectra of DNA fragments is difficult to make in terms of absolute structural features (12, 17). The interpretation of the CD spectra of the AAF monoadducted duplexes is further complicated by the extensive overlapping of the absorption spectra of AAF and DNA. As a consequence, the observed circular dichroïsm signal is the sum of the signal induced by the DNA structure itself and the circular dichroïsm signal induced in the fluorene residue. Indeed, in the vicinity of the helix, AAF adducts become optically active (induced circular dichroïsm). However, from the observation of the difference absorption spectra between DNA modified with N-AcO-AAF to various extents and unmodified DNA (16), it is possible to distinguish two regions : the 230−260 nm region in which the absorption of the -AAF adduct is minimal, and the 270−300 nm region where the absorption of the -AAF adduct is predominant. We will discuss the CD signal in the region of minimal influence of the fluorene ring (230−260 nm) in order to deduce qualitative features of the overall structure of the different duplexes.

The nonmodified duplex presents a typical B-form CD spectrum in agreement with the recent crystal structure of the same duplex (18). The first point to be stressed is the fact that all three modified duplexes present a substantially altered CD spectrum as compared to the non modified duplex (figure 4). To focus on the structural changes induced by the covalent binding of the -AAF adduct within this short B-DNA helix we will discuss the difference spectra between each monomodified duplex and the nonmodified duplex (figure 5). As seen from figure 5, all three modified duplexes have a different conformation showing a strong influence of the sequence in the vicinity of the guanine-AAF adduct. Qualitatively, the structure of the Nar-AAF1 duplex appears to be the least affected as compared to the non modified duplex. Similarly, the structure of the Nar-AAF2 duplex presents relatively little alteration of the CD signal in the region where the influence of the DNA is predominant (230−260 nm). In contrast, the difference spectrum (Nar-AAF3−Nar) suggests an important modification of the structure of the DNA helix (Figure 5). The similarity of this difference spectrum with a Z-form minus B-form differential spectrum is in favor of the coexistence of a left handed and right handed structure within this sequence. Such a possibility was suggested for $(dC.dG)_n.(dC.dG)_n$ randomly modified with AAF to a low extent (19). The coexistence of B and Z DNA within a short oligonucleotide was recently demonstrated (20, 21). An alternative explanation is that there is an equilibrium between molecules that are totally in the B form and molecules that are totally in the Z form. This putative equilibrium would be displaced toward the Z form when the salt concentration is increased. Since we do not see an increased Z DNA signal when increasing the ionic

strength to 3M NaCl (figure 6) we do not consider further this alternative possibility and favor the hypothesis that only part of the molecule adopts a Z-like structure upon modification with N-AcO-AAF.

Alternating purine-pyrimidine DNA sequences have been shown to undergo a B to Z transition when modified with N-AcO-AAF (14, 19, 22). This effect can be related to the rotation of the AAF-modified guanine bases from anti to syn conformation (26), which is the conformation of the deoxyguanosine in the Z-DNA structure (27). The B to Z transition has been observed for -AAF modified $(dC.dG)_n$.$(dC.dG)_n$ even at low salt concentration, and for $(dT.dG)_n$.$(dC.dA)_n$ under salt conditions that are insufficient to induce the B to Z transition in the non-modified polynucleotide (14, 19, 22). Unlike these sequences, the *NarI* site presents a purine-pyrimidine alternation over only 4 base pairs. However, modeling studies have shown that stretches as short as one dinucleotide dCpG can hold a Z-DNA conformation embedded within a B type helix (23).

In conclusion, modification by N-AcO-AAF of guanine G_1 or G_2 in the *NarI* site of the 12-mer d(ACCG$_1$G$_2$CG$_3$CCACA) induces local changes near the AAF adduct that do not change the general features of the B-DNA helix. In contrast, modification of guanine G_3 causes a major alteration in the local structure of the helix. The qualitative interpretation of the CD spectrum suggests the existence of a local Z-like DNA structure. High resolution NMR studies are presently under way to further elucidate the structural features of these modified duplexes. It has recently been shown that only binding of AAF to G_3 leads to the -2 frameshift event within the NarI mutation hot spot (7). We suggest that this specific -2 frameshift event results from the processing of the unusual DNA structure that is induced by AAF upon binding to G3. It is noteworthy to add that Z-forming DNA sequences have recently be found to be spontaneous deletion hot spots (24).

The use of DNA substrates having single, chemically well defined adducts makes it possible to investigate the influence of the local DNA sequence in the repair and mutagenic processing of these adducts. The *NarI* site has been discovered has a strong hot spot for -2 frameshift mutations induced by AAF (1, 2) and more generally by a variety of chemical carcinogens that bind to the C8 position of guanine (25). Single adduct studies have shown a strong dependence of the exact position of the -AAF adduct on both excision repair (28) and mutagenesis (7). The strong sequence effects described in this paper present the first structural data showing the polymorphic nature of the DNA structure in the vicinity of a given carcinogen adduct.

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