N-acetoxy-2-acetylaminofluorene modification of a deoxyoligonucleotide duplex

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ABSTRACT

The carcinogen N-acetoxy-2-acetylaminofluorene was reacted with d(CCACGCACC) to form a covalent adduct with attachment at the single guanine. The sample was purified, mixed 1:1 with d(GGTGCGTGG) and studied by thermal denaturation experiments. The T_m for the mixture was $35 \pm 3^\circ\text{C}$, consistent with duplex formation. The method of AAF continuous variation shows that the modified oligomer, d(CCACG^{AA}ACC), forms a 1:1 duplex with d(GGTGCGTGG). Circular dichroism spectra also indicate the formation of a duplex and suggest that the modified duplex has a left-handed conformation. Addition of the intercalating drug ethidium alters the CD spectrum of the modified duplex, resulting in a CD spectrum similar to that of ethidium bound to right-handed DNA.

INTRODUCTION

The reaction of the carcinogen N-acetoxy-2-acetylaminofluorene (AAAF) with DNA is well established (1,2). The primary product involves the formation of a covalent bond between the 2-amino moiety of 2-acetylaminofluorene (AAF) and the C-8 of guanine, as shown in Figure 1. The effect of this adduct on DNA conformation may have relevance to the mechanism of carcinogenesis (3) and is an area of active interest.

Two related models, termed insertion-denaturation and base displacement, have been proposed for the conformation of the DNA-AAF adduct. The insertion-denaturation model is based on a series of experiments with AAF modified DNA which suggest that the DNA is locally denatured at the site of AAF modification and that the fluorene moiety is inserted between the base pairs adjacent to the modified guanine (4-8). The base displacement model was proposed from model building studies which indicated that the guanine had a syn conformation, and from studies with

single-stranded oligonucleotide adducts (9-12). In the base displacement model the modified guanine is displaced from the helix with the AAF moiety stacked with the adjacent bases.

AAAF modification of poly(dG-dC) facilitates conversion of the polymer from B-form to a left-handed Z-form. When 6.6% of the guanines are modified the conversion required less ethanol than with unmodified polymer (13), and at 28% modification the polymer adopts the Z-form under low salt conditions, in the absence of ethanol (14). The facilitation of the B-form to Z-form transition has also been observed in AAF modified poly(dT-dG)·poly(dC-dA) (15).

The conformation of the AAF adduct in a left-handed helix probably differs from the conformation of the adduct in a right-handed helix. In Z-form poly(dG-dC), guanine has the syn conformation and the C-8 is located at the exterior of the helix (16), so base pairing need not be disrupted by AAF modification. Modified poly(dG-dC) in the Z-form was resistant to single-strand-specific endonuclease (14). The characteristics of AAF modified poly(dG-dC) led to the development of a model for the conformation of the AAF adduct in Z-DNA in which AAF is located at the exterior of the helix with little denaturation or interruption of base pairing at the site of modification (14).

AAAF modified nucleotides and single-stranded oligonucleotides have been studied as models for the DNA-AAF adduct. The conformation and dynamics of AAF modified deoxyguanosine and deoxyguanosine-5'-monophosphate have been examined by high resolution NMR (17-19). CD and NMR data are available on several single-stranded oligonucleotide adducts (10-12, 20-22). Several stable conformations of the DNA-AAF adduct have been proposed based on minimized potential energy calculations (23-25). In preliminary experiments (unpublished results) we have shown that the AAAF adduct with the self-complementary deoxyhexanucleotide d(ATGCAT) is formed readily but the AAF moiety sufficiently destabilizes the duplex that only single-stranded molecules are observed. Rio et al. (20) have reported similar results with self-complementary oligonucleotides in which all the guanines were modified. To obtain detailed conformational information relevant to AAF adducts in double-helical DNA a model system of

an AAF adduct with a stable deoxyoligonucleotide duplex is required. We have synthesized complementary strands of deoxyoligonucleotides containing 9 bases in which one strand was designed with a single guanine to provide a single site of attachment for AAF. In this manuscript we present the initial experiments on the conformation and stability of the modified and unmodified duplexes.

MATERIALS AND METHODS

The nonanucleotides d(CCACGCACC) and d(GGTGCGTGG) were synthesized on an Applied Biosystems DNA Synthesizer using the solid phase phosphoramidite method (26,27). Extinction coefficients at 260 nm were calculated to be $78,000 \text{ M}^{-1} \text{ cm}^{-1}$ for d(CCACGCACC) and $86,000 \text{ M}^{-1} \text{ cm}^{-1}$ for d(GGTGCGTGG), using the method of Cantor, Warshaw and Shapiro (28). Molar extinction coefficients for deoxyoligonucleotides are expressed in terms of strand concentrations.

N-Acetoxy-2-acetylaminofluorene (AAAF) was obtained from the National Cancer Institute Carcinogen Standard Reference Repository. Ethidium bromide was purchased from Sigma and purified by recrystallization from methanol. A molar extinction coefficient of $5680 \text{ M}^{-1} \text{ cm}^{-1}$ was used at 480 nm for ethidium.

AAAF was reacted with d(CCACGCACC) by adding a 5:1 excess of the carcinogen in 0.002 M sodium citrate buffer, pH 7, 20% ethanol. The reaction mixture was incubated for three hours at 37 °C. To terminate the reaction and remove unbound fluorene residues, the sample was extracted three times with an equal volume of diethyl ether. The major reaction product was separated from the unreacted oligonucleotide by reverse phase high performance liquid chromatography (HPLC). An Alltech C18 preparative HPLC column, 25 cm x 10 mm, was used to purify the adduct. A 0-30% acetonitrile gradient increasing at 1% per minute was used with a flow rate of 7 mL per minute. The buffer was 0.010 M ethylenediamine acetate, pH 7.

All UV and visible absorption measurements were made on a Varian Cary 219 UV/VIS spectrophotometer, except for the melting experiments described below. CD spectra were recorded on a Jasco J-40 spectropolarimeter interfaced to a Digital Equipment

Corporation PDP 11/34 computer. CD spectra were recorded by measuring the ellipticity between 320 nm and 240 nm, at 5 nm intervals. At each wavelength, 200 data points were collected, averaged, and baseline corrected.

In the melting experiments, the absorbance was monitored as a function of temperature in a Gilford model 250 spectrophotometer with a Gilford 2527 thermoprogrammer, interfaced to a Digital Equipment Corporation PDP 11/34 computer. The samples for all melting curves were in a buffer consisting of 1.0 M NaCl, 0.010 M sodium cacodylate, 0.1 mM EDTA, pH 7. Sample concentrations were $6.4 \mu\text{M}$ in strands; the initial absorbance was between 0.5 and 0.6 in a 1 cm pathlength cell. The temperature was raised from 0°C to 80°C at a rate of one degree per minute. The low temperature data and the high temperature data were fit to straight lines and the T_m was taken as the temperature at which the absorbance is halfway between the values calculated for these lines (29).

The method of continuous variation (30) was used to determine the stoichiometry of complex formation between $d(\text{CCACG}^{\text{AAF}}\text{CACC})$ and $d(\text{GGTGCGTGG})$. The absorbance at 260 nm was monitored as the mole fraction of each strand was varied, while keeping the total strand concentration constant. The data were fit to two straight lines; the intersection of these lines is the stoichiometric ratio of the complex.

RESULTS AND DISCUSSION

The structure of AAAF and a schematic illustration of the experiment described here are shown in Figure 1. The oligomer, $d(\text{CCACGCACC})$, has a single guanine and hence a single site for the formation of the major AAF adduct. Since the reaction was performed on the single stranded deoxyoligonucleotide, the guanine C-8 adduct was expected to be the only product (31). The covalent adduct, $d(\text{CCACG}^{\text{AAF}}\text{CACC})$, was separated from unreacted deoxyoligonucleotide by HPLC (Figure 2). Only one reaction product was detected by reverse phase HPLC; the ultraviolet absorption spectrum (Figure 2) of this product matches previously published spectra for the C-8 adduct (1,2). The 305 nm extinction coefficient for $d\text{G}^{\text{AAF}}$ is $18,000 \text{ M}^{-1} \text{ cm}^{-1}$ (2,32). We have assumed that the 305 nm extinction coefficient of the

Figure 1. Schematic representation of the reaction of AAF with the deoxyoligonucleotide d(CCACGCACC) and the formation of a duplex with the complementary strand. AAF reacts with the single guanine in d(CCACGCACC) to form a covalent adduct. The purified adduct is then mixed 1:1 with the complementary strand d(GGTGCGTGG) to form a modified duplex.

Figure 2. HPLC profile monitored at 254 nm of the AAF + d(CCACGCACC) reaction mixture after extraction with diethyl ether. Peak (a) co-elutes with d(CCACGCACC) at 9.5 minutes. The inset shows the ultraviolet absorption spectra of peaks (a) and (b) dissolved in 1.0 M NaCl, 0.010 M sodium cacodylate, 0.0001 M EDTA, pH 7.

Figure 3. Optical melting curves at 260 nm. Curve (a), d(GGTGCGTGG)·d(CCACG^{AAF}CACC); Curve (b), the unmodified duplex, d(GGTGCGTGG)·d(CCACGCACC). Samples are 6.4 μ M (strands) in 1.0 M NaCl, 0.010 M sodium cacodylate buffer with 0.0001 M EDTA, pH 7. The T_m for the modified duplex, (a), is 35 ± 3 °C and for the unmodified duplex, (b), the T_m is 53 ± 3 °C. Smooth lines were drawn through 200 data points recorded from 0 to 80 °C.

d(CCACG^{AAF}CACC) adduct at 80 °C is the same ($18,000 \text{ M}^{-1} \text{ cm}^{-1}$) as for dG^{AAF}. Using this we calculate the 260 nm extinction coefficient at 25 °C for the modified strand to be $82,000 \text{ M}^{-1} \text{ cm}^{-1}$. The extinction coefficient of $82,000 \text{ M}^{-1} \text{ cm}^{-1}$ for the adduct is slightly larger than the value of $78,000 \text{ M}^{-1} \text{ cm}^{-1}$ calculated for the unmodified oligonucleotide. This was expected since the AAF will have some absorbance at 260 nm (5).

Modification at the C-8 of guanine is known to destabilize the double helix (5). A plot of absorbance vs temperature for a 1:1 mixture of d(CCACG^{AAF}CACC) with its complementary strand d(GGTGCGTGG) (Figure 3) shows a cooperative transition on heating the sample from 0 to 80 °C. Comparing the melting curves at 260 nm for modified and unmodified samples shows that AAF modification decreases both the T_m and the hypochromicity. The T_m for the modified duplex was 35 ± 3 °C while for the unmodified duplex the T_m was 53 ± 3 °C. We also observed a cooperative transition at 305 nm for the modified sample (data not shown). This transition was not observed for the unmodified duplex or for

Figure 4. AAF Mixing curve for various ratios of d(GGTGCGTGG) and d(CCACG^{AAF}CACC) at a constant total strand concentration. The two lines drawn through the 260 nm absorbance data indicate a 1:1 stoichiometry.

the modified oligomer d(CCACG^{AAF}CACC) alone. The T_m for this transition, $35 \pm 3^\circ\text{C}$, was essentially the same as that obtained from 260 nm data.

The method of continuous variation was used to determine the stoichiometry of the complex between d(GGTGCGTGG) and d(CCACG^{AAF}CACC). The results are shown in Figure 4. It is clear that a complex forms which results in hypochromicity at 260 nm. The lines drawn through the data points intersect at an equimolar ratio of the two strands, indicating a 1:1 stoichiometry.

Adduct formation also resulted in major changes in the CD spectrum. The CD spectra of the modified and unmodified duplexes are shown as the bold, solid lines in Figure 5. The spectrum of the 1:1 d(CCACGCACC) : d(GGTGCGTGG) duplex is consistent with a right-handed (B-form) deoxyoligonucleotide with a high G·C content. The CD spectrum of the modified duplex is reminiscent of the CD spectrum exhibited by AAF modified poly(dG-dC) in the Z conformation (13,14). With an internal core of 7 alternating pyrimidine-purine bases in d(CCACGCACC)·d(GGTGCGTGG), a left handed conformation for the modified duplex is reasonable. The left-handed type of CD spectrum for the modified duplex is observed at a salt concentration of 0.10 M NaCl, which is well

Figure 5. CD spectra of the unmodified (A) and modified (B) duplexes with varying amounts of added ethidium. The spectra of the samples without ethidium are drawn with bold, solid lines. The numbers above selected spectra indicate the number of drugs added per duplex in that sample. Samples are in 0.1 M NaCl, 0.010 M sodium phosphate, 0.0001 M EDTA, pH 7. All spectra were recorded at 5 °C.

below the 3 M NaCl needed to produce the Z form in unmodified poly(dG-dC). At one AAF bound per 18 nucleotides the extent of modification is much lower than the 28% modification needed to invert the CD spectrum of poly(dG-dC) at an equivalent NaCl concentration. This may be due to end effects or perhaps is a function of the nucleotide sequence.

The CD spectra of the modified and unmodified duplexes with various ratios of ethidium per duplex are shown in Figure 5. This data shows that the 285 nm ellipticity of the modified duplex undergoes a smooth transition from a negative band to a positive band as the ethidium concentration is increased, in a manner similar to the ethidium induced Z-B transition of poly(dG-dC) in 4.4 M NaCl solution (33,34). The spectra of the modified and unmodified duplexes in the presence of ethidium are very

similar in the 240-280 nm region, which also supports the interpretation that the AAF duplex has a left-handed conformation in the absence of ethidium. There are some differences in the magnitude of the molar ellipticity in the 290-320 nm region of the spectra of the modified and unmodified duplexes in the presence of ethidium. Much of this difference may be due to contributions from the fluorene moiety which absorbs in this region (5). The CD spectrum of ethidium bound to DNA exhibits a band in the 310-330 nm region whose magnitude, normalized to the concentration of bound ethidium, increases as the degree of saturation increases (35). This band is observed in the titration of ethidium with the unmodified duplex, although the magnitude of the band is quite small until there are two ethidiums added per duplex. With the AAF modified duplex the ellipticity at 320 nm is apparent at relatively low levels of ethidium (0.2 drugs per duplex) which may reflect the effect of the AAF moiety on the conformation of the ethidium binding sites. A distortion of the right-handed helix would be expected if the modified duplex has a conformation similar to either the base displacement or insertion-denaturation models.

We have shown with thermal denaturation experiments and continuous variation experiments that d(CCACG^{AAF}CACC) and d(GGTGCGTGG) form a stable duplex. The T_m 's of the modified and unmodified duplexes illustrate the destabilizing effect of adduct formation by AAF. The 305 nm thermal melting data suggest an ordered structure for the fluorene residue with the possibility of stacking between the fluorene ring and the bases. CD studies indicate that the modified duplex has a left-handed conformation which is converted to a right-handed helix on binding ethidium. Proton NMR studies now in progress should provide detailed information concerning the conformation of the carcinogen-DNA adduct.

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