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DNA Conformation Mediates Aflatoxin B_1-DNA Binding and the Formation of Guanine N^7 Adducts by Aflatoxin B_1 8,9-*exo*-Epoxide

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The binding of aflatoxin B_1 to DNA and DNA adduction by aflatoxin B_1 *exo*-8,9-epoxide were studied as a function of DNA conformation. Equilibrium binding of aflatoxin B_1 to A-, B-, and Z-form helices was monitored by measurement of NMR linewidth for the methoxy protons of aflatoxin B_1. The data revealed that as compared to B-form DNA, the association of aflatoxin with the A-form helix is significantly reduced. No binding to Z-DNA was observed. The yields obtained following reaction of the respective monomers with aflatoxin B_1 *exo*-8,9-epoxide revealed that only dG forms an adduct. The reactivity of aflatoxin B_1 *exo*-8,9-epoxide with single-strand DNA was determined via constant-temperature experiments in which the yield of adduct was measured for a family of oligonucleotides of increasing thermal stability. The results demonstrate that duplex structure favors adduct formation. Adduct yields were compared for A-, B-, and Z-form helices. About 12 times less adduct is produced from the A-form helix as compared to B-form, while no adduct is produced from a Z-form duplex. The results support the conclusion that reaction of aflatoxin B_1 *exo*-8,9-epoxide with DNA proceeds via an intercalated transition-state complex only with the B-form double helix.

The aflatoxins are a group of structurally related mold metabolites containing a dihydrofuran moiety attached to a coumarin ring system (*1*). The fungi infest a variety of human foodstuffs including corn and peanuts. Aflatoxin B_1 is a mutagen in several tester strains of bacteria (*2*). It is a hepatocarcinogen in experimental animals (*1*, *3-5*), and epidemiological studies suggest that it causes cancer in humans as well (*1*, *6-9*). Aflatoxin may be linked to site-specific transversions in the tumor suppressor gene p53 (*10*, *11*).

Aflatoxin B_1 requires metabolic activation to exact its biological effects. The carcinogenic effects of aflatoxin B_1 are believed to be initiated by adduction of aflatoxin B_1 *exo*-8,9-epoxide to DNA (Figure 1). The isomeric aflatoxin B_1 *endo*-8,9-epoxide has also recently been isolated, but its genotoxicity and mutagenicity have not been established (*12*). Several specific cytochromes P450 oxidize aflatoxin B_1 in humans (*13-15*). Reaction occurs at the N^7 of guanine to give *trans*-8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 (*16*). We have found substantial evidence for intercalation of aflatoxins B_1 and G_1 into the B-DNA helix (*17*, *18*). We have further demonstrated that, for two oligodeoxynucleotide adducts of aflatoxin B_1, the aflatoxin moiety intercalates on the 5′ side of the guanine with which it reacts (*19*) and that alteration of the cyclopentenone ring of aflatoxin B_1 to a δ-lactone reduces binding affinity and the yield of adducts at guanine N^7 (*18*). We have proposed that intercalation is involved in the transition state of the adduction reaction and is necessary for adduction to occur (Figure 2) (*17*, *20*).

The present work examines aflatoxin B_1 binding to DNA and DNA adduction by aflatoxin B_1 *exo*-8,9-epoxide as a function of DNA conformation. The results support the conclusion that reaction of aflatoxin B_1 *exo*-8,9-epoxide with DNA proceeds via an intercalated transition-state complex only with the B-form double helix.

Experimental Section

Materials. Deoxyribonucleic acid from calf thymus was obtained from Sigma Chemical Co. (St. Louis, MO). The heteropolymer, poly(dGdC)·poly(dGdC), the copolymers, poly(dG)·poly(dC) and poly(rC)·poly(dG)$_{12-18}$, and the homopolymers, poly(dG) and poly(dI), were purchased as sodium salts from Pharmacia-LKB Biotechnology (Piscataway, NJ). The decadeoxynucleotide 5′-DMT-d(TATACGTATA)-3′-OH was kindly provided by Dr. Richard Hodge at the MilliGen/Biosearch Division of Millipore Corp. (Burlington, MA). The hexadeoxynucleotide, d(TACGTA), and the tetradeoxynucleotide, d(TCGA), were prepared by standard solid-phase phosphoramidite chemistry. The deoxynucleosides, 2′-deoxyguanosine, 2′-deoxyadenosine, 2′-deoxycytidine, and thymidine, were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Aflatoxin B_1 was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Aflatoxin B_1 is a potent carcinogen. **Caution**: *Crystalline aflatoxins are particularly hazardous due to their electrostatic nature and should be handled using appropriate containment procedures and respiratory mask to prevent inhalation. Aflatoxins can be destroyed by treatment with NaOCl.*

Measurements of Excess ^{1}H NMR Linewidth. An A-form duplex was obtained by preparing the DNA·RNA hybrid polymer poly(rC)·poly(dG)$_{12-18}$ in 15 mM sodium phosphate buffer (pH 7.4). Z-Form DNA was generated by addition of 130 μM Co(NH$_3$)$_6$ to the alternating copolymer poly(dGdC)·poly(dGdC) in 15 mM sodium phosphate buffer (pH 7.4) (*21*). A Cary Model 60 recording spectropolarimeter was used for circular dichroism measurements. A stock solution (2.5 mM) of aflatoxin B_1 was prepared in dimethyl-d_6 sulfoxide. Ten microliters of stock was added to 400 μL of D_2O buffer [0.1 M NaCl, 5×10^{-5} M Na_2-EDTA, and 0.01 M sodium phosphate buffer (pH 7.0)] to make a 61 μM NMR sample. Stock solutions of 0.22 and 1.05 mM calf thymus DNA in D_2O buffer containing 61 μM aflatoxin were prepared by addition of aflatoxin stock solution. The aflatoxin NMR sample was titrated with the DNA-aflatoxin mixture, with

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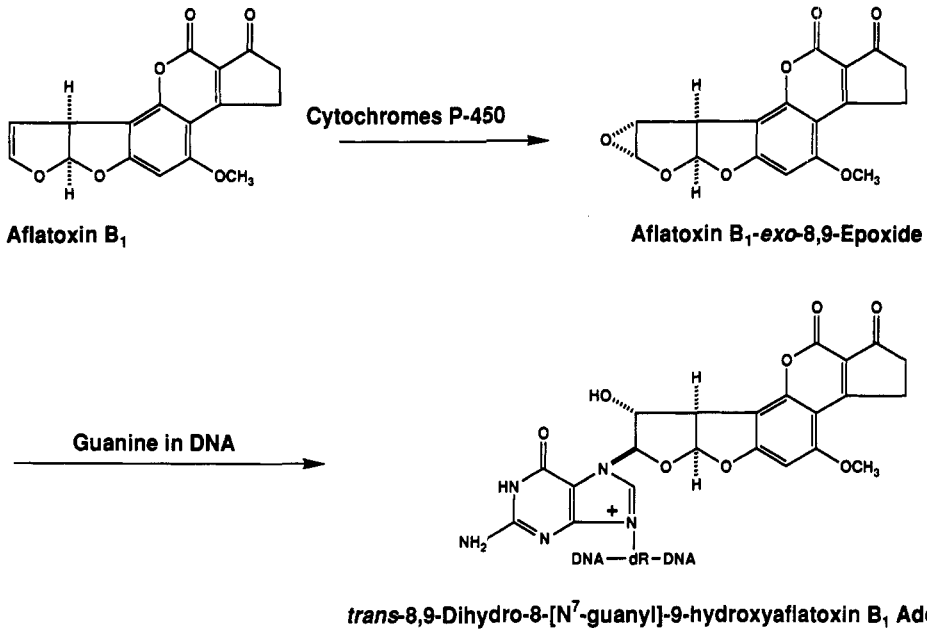

Figure 1. Metabolic activation of aflatoxin B_1 to aflatoxin B_1 *exo*-8,9-epoxide is carried out by cytochrome P450. The epoxide reacts regio- and stereoselectively with N^7 of guanine to form the primary adduct *trans*-8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1.

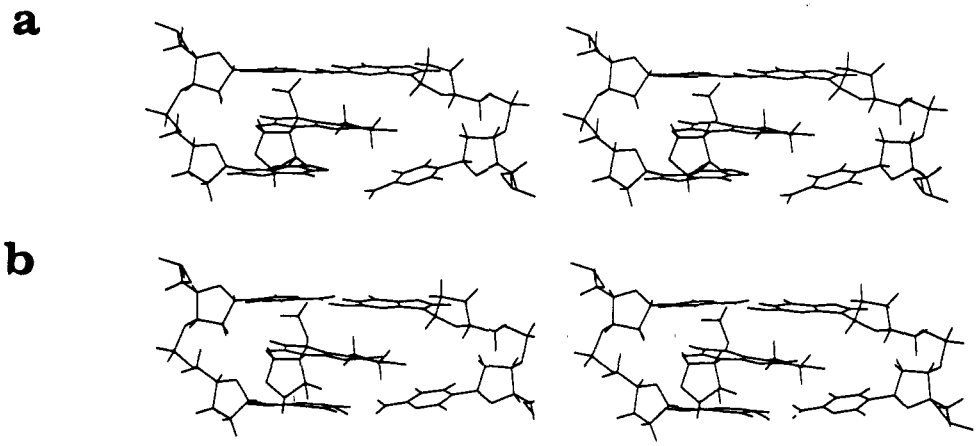

Figure 2. Proposed mechanism for the adduction of guanine N^7 by aflatoxin B_1 *exo*-8,9-epoxide involves the (a) initial intercalation of the epoxide above the 5′ face of guanine, thus positioning the epoxide for attack by N^7 to yield the intercalated cationic adduct (b). For purposes of illustration, we have positioned the intercalated epoxide and the subsequent adduct at the guanine N^7 adduct within the dimer $d(CpG)_2$. The molecular modeling was done with INSIGHTII/DISCOVER (Biosym Technologies, Inc., San Diego, CA), using the AMBER forcefield (*44*), without incorporation of experimentally derived constraints.

gentle mixing after each addition of DNA. After each addition of DNA, a 400.13-MHz ^1H NMR spectrum was obtained at 27 ± 1 °C. Measurement of DSS linewidth served as a check for magnetic field homogeneity and changed by less than 0.5 Hz from sample to sample. The linewidth of the methoxyl peak was measured at increasing concentrations of DNA.

Reaction of Aflatoxin B_1 *exo*-8,9-Epoxide with Nucleic Acids. Aflatoxin B_1 *exo*-8,9-epoxide was prepared by using dimethyldioxirane (*22*), which typically yields a 10:1 exo:endo ratio of epoxides (*12*); the *exo*-epoxide can be crystallized using CH_2Cl_2/acetone (1:1 v/v). **Caution**: *It should be assumed that aflatoxin epoxides are highly toxic and carcinogenic. Manipulations should be carried out in a well-ventilated hood with suitable containment procedures.* The concentration of aflatoxin B_1 *exo*-8,9-epoxide was determined spectrophotometrically using an extinction coefficient of 21 800 M^{-1} cm^{-1}. Nucleic acid samples were prepared as 33 μM guanine and 33 μM aflatoxin B_1 *exo*-8,9-epoxide in 10 mM sodium phosphate buffer (pH 7.4). Calf thymus DNA was made 83 μM by base pairs of which 40% were guanine. The concentrations of poly(dGdC)·poly(dGdC), poly(dG)·poly(dC), and poly(rC)·poly$(dG)_{12-18}$ were determined in base pairs which equaled the concentration of guanine. Concentrations for poly(dG), poly(dI), d(TATACGTATA), d(TACGTA), and d(TCGA) were calculated as single strand which was the same as the concentration of guanine. Extinction coefficients were calculated as single-stranded values (*23*) and then corrected for pH. A Varian Cary 2390 UV–vis–near-IR spectrophotometer interfaced with a Neslab ETP-3 temperature programmer unit controlling a Neslab Endocal refrigerated circulating bath was used for UV spectroscopy. Deoxyguanosine, deoxyadenosine, deoxycytidine, and thymidine were prepared as 1.1 mM solutions in 5% Me_2SO in 10 mM sodium phosphate buffer (pH 7.4). Aflatoxin B_1 *exo*-8,9-epoxide was added to a concentration of 50 μM. All reactions were performed under subdued light to minimize potential formation of aflatoxin B_1 photoproducts or photodecomposition of the resulting carcinogen–DNA adduct (*24–27*).

Results

Equilibrium Binding of Aflatoxin B_1 to A-, B-, and Z-Form Polymers. The association of aflatoxin B_1 with calf thymus DNA, poly(dAdT)·poly(dAdT), and poly(dGdC)·poly(dGdC) in 0.1 M NaCl/0.1 M sodium phosphate buffer (pH 7) was previously demonstrated to be of similar magnitude (*20*, *26*); typical association constants as measured by Scatchard analysis of equilibrium dialysis

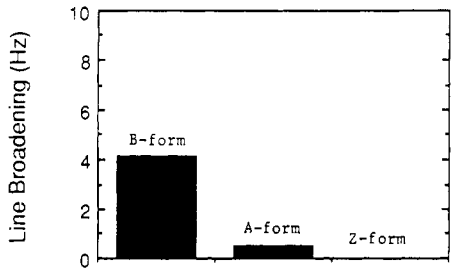

Figure 3. Equilibrium binding of aflatoxin B_1 as a function of DNA duplex conformation measured as increased line broadening of the aflatoxin B_1 methoxy protons. B-Form calf thymus DNA, A-form poly(rC)·poly(dG)$_{12-18}$, and Z-form poly(dGdC)·poly(dGdC) were examined.

data were 1.5×10^3 M^{-1}. Likewise, Scatchard analysis of NMR data for the oligonucleotide d(ATGCAT)$_2$ in 0.1 M NaCl buffer yielded a K_a of 3.7×10^3 M^{-1} (*17*). The NMR linewidth of the aflatoxin B_1 methoxyl resonance in the absence of DNA is 1.6 ± 0.1 Hz. Calf thymus DNA in 10 mM sodium phosphate buffer (pH 7.4) forms a right-handed B-type helix as monitored by ultraviolet circular dichroism. Addition of 33 μM calf thymus DNA under these conditions results in an increase of 4-Hz line broadening of the aflatoxin B_1 methoxy protons (*18*), consistent with association between aflatoxin B_1 and B-DNA (Figure 3).

A small, but measurable increase in linewidth of the aflatoxin B_1 methoxyl protons from 1.6 ± 0.1 to 2.1 ± 0.1 Hz was observed upon addition of 33 μM A-form poly(rC)·poly(dG)$_{12-18}$. A control experiment performed under the same conditions but substituting calf thymus DNA for poly(rC)·poly(dG)$_{12-18}$ showed a corresponding increase in linewidth for the methoxy protons to 5.7 Hz. The data revealed that as compared to B-form DNA the association of aflatoxin B_1 with the A-form helix is significantly reduced.

No line broadening of the aflatoxin B_1 methoxy resonance was detected upon addition of Z-form poly(dGdC)·poly(dGdC) to aflatoxin B_1. The final concentration of poly(dGdC)·poly(dGdC) which was achieved was 35 μM, with the linewidth of the aflatoxin B_1 methoxyl protons remaining at 1.6 ± 0.1 Hz. A control experiment in which the methoxyl linewidth of aflatoxin B_1 was monitored in the presence of 130 μM $Co(NH_3)_6Cl_3$ in 15 mM sodium phosphate buffer (pH 7.4) demonstrated that this concentration of $Co(NH_3)_6Cl_3$ did not cause line broadening. An additional control experiment in which poly(dGdC)·poly(dGdC) was replaced with calf thymus DNA which does not undergo the B–Z transition demonstrated that $Co(NH_3)_6Cl_3$ reduces but does not prevent the association of aflatoxin B_1 with B-form DNA.

Adduct Formation by Aflatoxin B_1 as a Function of DNA Conformation. (a) Monomers and Single-Strand DNA. The yields obtained following reaction of the respective monomers with aflatoxin B_1 8,9-*exo*-epoxide revealed that only dG forms an adduct; this adduct was obtained in 5.8% yield. The reactivity of aflatoxin B_1 8,9-*exo*-epoxide with single-strand DNA was determined via constant-temperature experiments in which the yield of adduct was measured for a family of oligodeoxynucleotides of increasing thermal stability. At 30 °C, both d(TCGA) and d(TACGTA) are single stranded while d(TATACGTATA) is substantially double stranded. d(TATACGTATA) has a melting temperature of 37.7 °C; d(TCGA) has a $T_m \leq 10$ °C. The yields of 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 obtained for these oligomers at this temperature, ~2% for d(TCGA) and d(TACGTA) and 27% for d(TATACGTATA), confirm that duplex structure favors adduct formation.

Table I. Percent Yields of *trans*-8,9-Dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 as a Function of DNA Conformation[a]

nucleic acid	conformation[b]	% yield[c]
calf thymus DNA	B-form	18 ± 2
poly(dGdC)·poly(dGdC)	B-form	13 ± 2
poly(dG)·poly(dC)	B-form	12 ± 2
calf thymus DNA + 130 μM $Co(NH_3)_6Cl_3$	B-form	7.0 ● 3
poly(dGdC)·poly(dGdC)	Z-form	negligible[d]
poly(rC)·poly(dG)$_{12-18}$	A-form	1.0 ± 0.5
poly(dG)	not determined[e]	2.0 ± 1
poly(dI)	not determined[e]	negligible[d]
d(TATACGTATA)	ss–ds equilibrium[f]	27 ± 2
d(TACGTA)	ss–ds equilibrium[f]	2.0 ± 1
d(TCGA)	ss–ds equilibrium[f]	2.0 ± 1

[a] Reaction conditions are summarized in the text. [b] Conformation was verified by circular dichroism. [c] Percent (%) yield of *trans*-8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 calculated as a percentage of aflatoxin B_1 8,9-*exo*-epoxide added to the reaction mixture. Each value represents an average from duplicate reaction mixtures; some samples were reacted in triplicate. For each reaction mixture, HPLC analysis was performed in duplicate. The range of each result is indicated. [d] In these instances, no more than 0.5% yield could be obtained. The detection limit was 0.1%. [e] These homopolymers were expected to equilibrate between single-strand and multiple-stand species under the reaction conditions. [f] These oligonucleotides equilibrate between single-strand and B-form duplexes. Only d(TATACGTATA) is substantially double stranded under the reaction conditions.

(b) Bonding as a Function of Duplex Conformation. To examine reactivity with an A-form duplex, poly(rC)·poly(dG)$_{12-18}$ was compared with poly(dG)·poly(dC). About 12 times less adduct is produced for the A-form DNA–RNA hybrid (Table I). Poly(dGdC)·poly(dGdC) was used to compare reactivity of B-form vs Z-form DNA with aflatoxin B_1 8,9-*exo*-epoxide. The yield of adduct for Z-form poly(dGdC)·poly(dGdC) was judged to be negligible, occurring with a yield of <0.5%, which may have arisen from the small fraction of B-form DNA in equilibrium with the Z-form. A control experiment using calf thymus DNA, with and without the salt, plus aflatoxin B_1 8,9-*exo*-epoxide was performed to discount the possible inhibition of 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 formation by $Co(NH_3)_6Cl_3$. The results of the control reactions revealed a 2-fold decreased yield of adduct, which was consistent with the NMR line broadening experiments which indicate that $Co(NH_3)_6Cl_3$ reduces DNA binding affinity.

Discussion

A major goal of this research program has been to develop an understanding of the properties which make aflatoxin B_1 *exo*-8,9-epoxide ideally suited to forming DNA adducts. Aflatoxin B_1 has a relatively low binding affinity for DNA, and the binding affinity of aflatoxin B_1 8,9-*exo*-epoxide is expected to be correspondingly low. The half-life of this epoxide is not known but is estimated to be on the order of seconds in aqueous solution. This epoxide reacts readily with DNA, both regiospecifically and stereospecifically, which suggests that adduct formation is directed by binding of this epoxide to the DNA helix with a specific orientation which promotes formation of a favorable transition state (*17*, *27*, *28*).

Molecular modeling reveals that intercalation of aflatoxin B_1 *exo*-8,9-epoxide above the 5′ face of guanine

positions aflatoxin B_1 *exo*-8,9-epoxide in an orientation to facilitate endo attack by the nucleophile followed by trans opening of the epoxide (*29*, *30*). This would account for the observed efficiency of adduct formation by aflatoxin B_1 *exo*-8,9-epoxide. Previous work from this laboratory provided experimental evidence for an intercalative transition state between aflatoxin B_1 *exo*-8,9-epoxide and B-DNA (*17–19*).

If adduction proceeds via an intercalative transition state involving B-DNA (Figure 2), then DNA conformation should play an important role in mediating binding and adduct formation. Systematic reaction of aflatoxin B_1 *exo*-8,9-epoxide with each of the monodeoxynucleotides yields only 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 which arises from reaction with deoxyguanosine. However, the reaction with deoxyguanosine proceeds with the low yield of 5.8% as compared to typical yields of 12–18% for guanine in B-form DNA under the same conditions (Table I). Experiments in which the epoxide was reacted with the oligodeoxynucleotides d(TCGA) and d(TACGTA), in comparison with d(TATACGTATA) which is substantially double stranded under the same conditions, reveal that double-stranded conformation strongly favors adduct formation at guanine N^7. We also examined the reactivity of aflatoxin B_1 *exo*-8,9-epoxide with the homopolymers poly(dG) and poly(dI) and observed it to be low. These polymers probably exist in equilibrium with multiple-strand species under the reaction conditions because the solution is at neutral pH and the salt concentration is low (*31*, *32*). Thus they may not be adequate models for guanine in single-strand DNA. Evaluation of the actual conformations these homopolymers assumed under the conditions of our experiments was not attempted. In any case, formation of duplex or quadraplex structures involving guanine N^7 would hinder or prevent reaction with the epoxide.

Among the three forms of duplex structures studied, neither A-form DNA–RNA hybrid nor Z-form DNA provided a good substrate for aflatoxin B_1 8,9-*exo*-epoxide. It was possible to isolate 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 from the A-form polymer, but the yield was only 1%, as compared to typical yields of 12–18% for B-form DNA under the same conditions. The low yield of adduct observed with A-form DNA is illustrative of the crucial role conformation plays in the interaction of this epoxide with the DNA. A priori, one might predict the right-handed A-helix, while conformationally distinct from B-DNA, might be sufficiently similar to enable comparable levels of aflatoxin binding and adduct formation. However, the NMR line broadening experiment for aflatoxin B_1 in the presence of A-form polymer reveals a reduced binding affinity. From this datum we cannot be certain that the binding to A-form is the result of intercalation. However, the A-form helix is perhaps not a good candidate for intercalation of aflatoxin B_1 *exo*-8,9-epoxide. The deeper and narrower major groove is less accessible (*33*), which may hinder formation of the intercalated species. One can also speculate as to whether altered base stacking overlaps in A-form vs B-form result in a less effective stacking geometry between the intercalated epoxide and the DNA.

The Z-helix might be expected to provide a good substrate for reaction with the epoxide, due to the convex major groove of Z-form presenting the N^7 atom for easy access (*34*, *35*). This, however, is not observed. The results of ^1H NMR line broadening experiments with Z-form DNA revealed no binding of aflatoxin B_1. The absence of any line broadening of aflatoxin B_1 after addition of aflatoxin B_1 8,9-*exo*-epoxide to Z-form poly(dGdC)·poly(dGdC) demonstrate that this epoxide does not interact with Z-DNA either noncovalently or covalently, consistent with the hypothesis that the transition state for the reaction requires an intercalated B-DNA complex. We are aware of only one report of ligand intercalation into Z-DNA (*36*), which seems to be a poor substrate for classical DNA intercalators. The interaction of ethidium bromide with Z-DNA is revealing in that no binding is observed at ethidium concentrations below 20 μM (*37*). Above this concentration, ethidium drives a cooperative conformational transition from the Z-helix to a right-handed helix into which the ethidium is intercalated (*37*, *38*). This phenomenon is not observed for aflatoxin B_1, presumably because the low aqueous solubility of aflatoxin B_1 prevents the concentration of free aflatoxin B_1 in equilibrium with Z-DNA from reaching a sufficient level. Adducts of aflatoxin B_1 at guanine N^7 stabilize the B-form helix and severely inhibit formation of Z-helices in poly(dGdC)·poly(dGdC) (*39*).

In summary, the available evidence indicates that guanine N^7 located within a region of B-form DNA provides the only major site for aflatoxin B_1 *exo*-8,9-epoxide reactivity in the DNA helix. Nevertheless, one cannot discount the possibility that undetected minor adducts in DNA might have significant genotoxicity. It is interesting to note that in vitro reactivity assays reveal guanine adduction levels to be sequence-specific (*27*, *40–43*). In sequences capable of forming hairpin loop structures, base-paired guanines reacted preferentially (*27*), which is corroborated by the present results. Thus, sequence-specific, microscopic conformational heterogeneity of the DNA helix might in part explain previous observations that not all guanines in DNA are equally reactive toward aflatoxin B_1 epoxide.

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