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CONFORMATIONS OF ADDUCTS AND KINETICS OF BINDING TO DNA OF THE OPTICALLY PURE ENANTIOMERS OF ANTI-BENZO(a)PYRENE DIOL EPOXIDE

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Kinetic flow dichroism studies indicate that the (+) enantiomer of 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene physically bound at intercalative-type sites in double-stranded DNA undergoes covalent binding reactions to form adducts at external binding sites. The conformation of the non-covalent complex derived from the (-) stereoisomer is also intercalative in nature, but the conformations of the covalent adducts are heterogeneous and are characterized by both intercalative-type and external conformations. It is suggested that the distinctly higher biological activity of the (+) enantiomer relative to the activity of the (-) enantiomer may be related to the preponderance of 7,8,9-triol benzo(a)pyrene residues covalently linked to deoxyguanine and located at external binding sites in the DNA adducts.

The ultimate mutagenic and tumorigenic form of the environmental pollutant benzo(a)pyrene (BaP) is the metabolite 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene or (+)anti-BaPDE (1,2). Its mirror image, the (-) enantiomer, displays a significantly lower order of biological activity either as a mutagen (3,4), or as a tumorigen in mice (5,6). It is generally believed that the covalent binding of BaPDE to cellular DNA constitutes the critical initial event which ultimately gives rise to mutations or tumor initiation (1-3). While there are significant differences in the reactivities of (+)anti BaPDE and (-)anti BaPDE with respect to double-stranded DNA (level of covalent binding of diol epoxide molecules to the DNA bases), the (+) enantiomer being more reactive than the (-) enantiomer (7,9), the differences in biological activities are not quantitatively correlated in any simple manner to the extent of covalent binding (3,9). Brookes and Osborne (3) have suggested that differences in the spatial orientation of the dominant adduct (the BaP-7,8,9-triol residue covalently linked at the 10-position with the exocyclic amino group of deoxyguanine), may account for the differences in the biological activities of the two enantiomers of anti BaPDE.

In order to examine this possibility and to investigate differences in reactivity of these two enantiomers with double-stranded DNA, we have studied the conformations of the non-covalent and covalent adducts formed between (+)

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or (-)anti-BaPDE and calf thymus DNA in aqueous solutions. Utilizing a flow linear dichroism technique (10) the changes in the conformations of the diol epoxide molecules can be followed as the physically bound BaPDE molecules undergo covalent binding to DNA.

EXPERIMENTAL

Calf thymus DNA (Worthington Biochemicals,Freehold, NJ) was purified as described previously (11). The hyperchromicity of this DNA was in the range of 38-40%. Stock solutions containing the desired quantity of DNA in 5mM Tris buffer were prepared, and small aliquots of BAPDE in tetrahydrofuran were added to these solutions. The progress of the reactions at 25°C was followed by monitoring the linear dichroism of these solutions as a function of time. At the end of the reaction (diol epoxide completely converted to either covalent DNA adducts or the tetraols BaPT (7,8,9,10-tetrahydrotetrahydroxybenzo(a)pyrene), the solutions were extracted twelve times with buffer-saturated ether to remove the BaPT molecules non-covalently bound to the DNA (12), and the flow dichroism of the covalent adducts was determined. The synthesis of the two enantiomers of anti-BaPDE was accomplished by a method described previously (13).

Linear dichroism spectra were measured utilizing a Couette flow cell consisting of a rotating, 29 mm diameter solid cylindrical suprasil rod sitting in a stationary cylindrical suprasil cell. The DNA solution was contained within the 0.7mm gap of this outer cylinder and the rotating inner cylinder. All experiments described here were performed at 1000 rpm which is sufficient to attain a maximal degree of orientation of the DNA molecules with the helical axis tending to align itself along the direction of the flow field (10). The dichroism ΔA is probed with a linearly polarized light beam incident at 90° with respect to the axis of the rotating cylinder and is defined by

$$\Delta A = A_{//} - A_{\perp} \propto A(3 \cos^2 \theta - 1) \qquad (1)$$

where $A_{//}$ and $A_{\perp}$ are the absorbances measured with the polarizer oriented parallel and vertical with respect to the flow direction. It is important to note that ΔA is proportional to A, the absorbance of the sample. The angle θ defines the direction of the transition moment of the pyrenyl chromophore with respect to the flow direction. This transition is oriented parallel to the long axis of the pyrene ring system (14). The ΔA signals can be either positive or negative, and two types of conformations have been characterized for BaP metabolite non-covalent and covalent adducts (15,16). Site I is characterized by a 10 nm red shift from 343-344 nm (free BaPDE or BaPT) to 353-354 nm upon binding of these molecules to DNA, and ΔA is negative. Within the absorption band of DNA (<300 nm), ΔA is also negative since the axis of the double helix tends to line up parallel to the orienting flow field (10). The conformation of site I is therefore consistent with an intercalative type of adduct structure in which there is significant overlap and interaction between the π-electrons of the pyrene ring system and the DNA bases. Site II is characterized by a small red shift to 345-346 nm in the absorbance due to the pyrenyl residue, and the linear dichroism signal ΔA is positive in sign. This conformation is consistent with that of an external adduct structure (14).

RESULTS AND DISCUSSION

Typical linear dichroism spectra measured at different time intervals after adding (+) or (-) anti-BaPDE to solutions containing DNA (7.5×10^{-6} M nucleotide concentration) are shown in fig. 1. This low concentration of DNA (as well as the high pH of 9.2) were selected in order to slow down the rate of reaction of BaPDE (17). About 1.5 minutes were required to assemble the

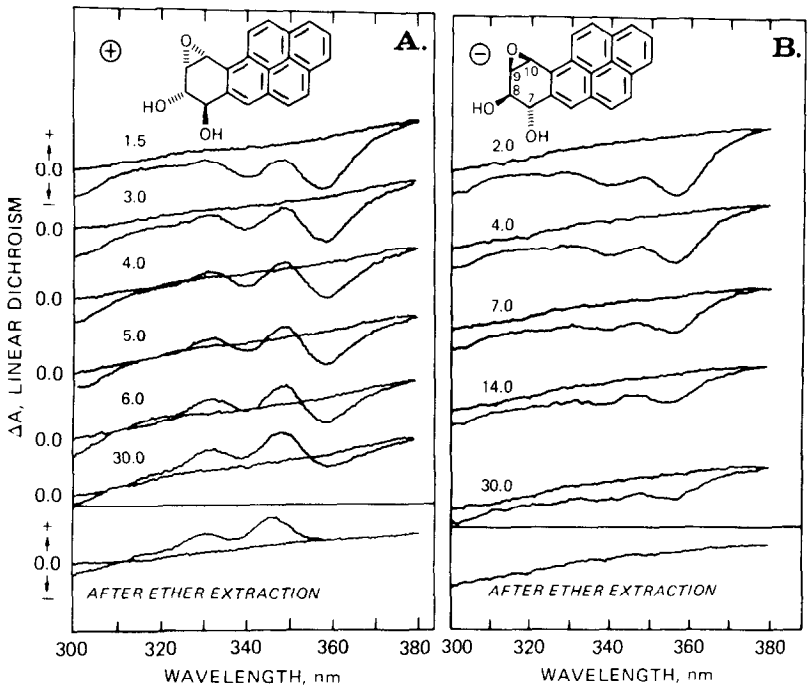

Figure 1. Linear dichroism spectra (in relative units) of reaction solutions (8.6×10^{-6} M anti-BaPDE, 7.6×10^{-5} M DNA in 5mM Tris buffer pH 9.2, 25°C) as as a function of time (indicated in minutes) after adding the diol epoxides to the solutions. The base lines, obtained without orientation of the DNA by flow, define the $\Delta A=0$ signal. (A) (+) enantiomer. (B) (-) enantiomer. After ether extraction: dichroism of covalently bound adducts; in (B) this signal is not distinguishable from the base line. The instrument sensitivity settings were the same in all experiments.

flow cell and to scan a full spectrum in the 300-380 nm wavelength interval (this scanning required ~15 seconds). The linear dichroism spectra exhibit variations as a function of time, but stabilize after about 10 minutes (fig. 2A). Typical equilibrium spectra 30 minutes after mixing are shown in fig. 1. The time dependent changes in the ΔA spectra may be understood in terms of the following:

(1) Immediately upon mixing (within 5 ms) non-covalent complexes between BaPDE and DNA are formed (11). The initial ΔA spectra in fig. 1 resemble the inverted absorption spectra of non-covalent complexes (15), and are consistent with site I, intercalative type of structures.

(2) With increasing time, more and more BaPDE molecules are hydrolyzed to tetraols (90-95% of all molecules initially added (12)) and undergo a covalent binding reaction with DNA (5-10% of all BaPDE molecules).

(3) The tetraols also form physical, site I type complexes with DNA (15), but with a smaller association constant than the diol epoxides (11), and account for the small negative linear dichroism signal around 360nm in the 30 min ΔA spectrum ((+) enantiomer) and for the full negative ΔA signal in the 30 min spectrum of the (-) enantiomer(figures 1A and 1B,respectively).

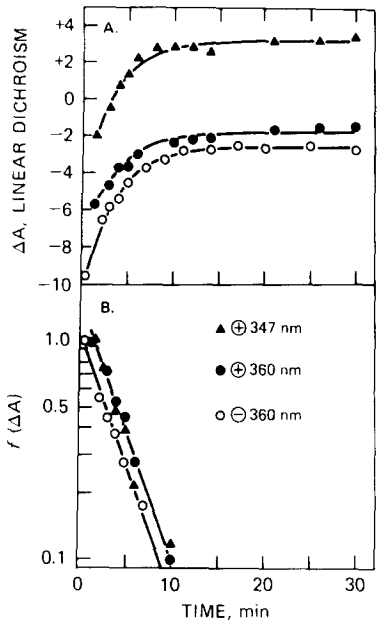

Figure 2. (A) Linear dichroism signal measured at 347nm (▲) and 360 nm in the case of the (+) enantiomer, and at 360nm (○) in the case of the (-) enantiomer. The solid lines are plots of eq. (2) with $k=3.6min^{-1}$. (B) Plots of eq. (3) showing that both enantiomers react with the same pseudo-first order rate constant $k=3.6\pm0.1\ min^{-1}$.

(4) Upon ether extraction, the non-covalently bound tetraols are removed and the linear dichroism spectra are uniquely due to the covalent adducts. For the (+) enantiomer, the ΔA spectrum is positive with maxima at 330 and 346 nm (as observed with electric field orientation of similar covalent adducts (14,18), and is characteristic of a site II external adduct structure. In the case of the (-) enantiomer, the ΔA signal is not significantly different from the base line (fig. 1B); the level of covalent binding of the (-) enantiomer is much lower than that of the (+) enantiomer (7,8) and thus cannot be discerned by the linear dichroism method under these particular conditions of low DNA concentration and instrument sensitivity.

(5) At intermediate reaction times (3-6 min spectra) in the case of the (+) enantiomer, the ΔA spectra are superpositions of covalently bound 7,8,9-triol BaP residues and non-covalently bound diol epoxide and tetraol molecules. With increasing time, the positive contribution of the covalently bound adducts at 345-350nm increases, while the negative contribution of the diol epoxide molecules above 350 nm decreases (fig. 1A). In the case of the (-) enantiomer (fig. 1B) only the physically bound diol epoxide and tetraol molecules contribute to the ΔA spectra to any significant extent.

The kinetics of these reactions for the (+) enantiomer can be further analyzed to demonstrate that the disappearance of intercalated BaPDE molecules is directly proportional to the rate of appearance of covalent adducts. Since

ΔA is proportional to A, and thus to the number of molecules bound to the DNA molecules, the kinetics of the disappearance of BaPDE (pseudo-first order rate costant k (17,19)) is proportional to $\Delta A_o e^{-kt}$, where ΔA_o is the initial value of the dichroism signal. The kinetics of appearance of products (covalent adducts + non-covalently bound BaPT) follows the expression $\Delta A_\infty(1-e^{-kt})$ where ΔA_∞ is the equilibrium value ($t\rightarrow\infty$) of the linear dichroism signal. The time dependent linear dichroism signal is thus a superposition of these two contributions and is:

$$\Delta A(t) = \Delta A_o e^{-kt} + \Delta A_\infty(1 - e^{-kt}) \qquad (2)$$

Upon rearranging this equation one obtains:

$$f(\Delta A) = (\Delta A(t) - \Delta A)/(\Delta A_o - \Delta A_\infty) = e^{-kt} \qquad (3)$$

Semi-logarithmic plots of this function for both enantiomers at wavelengths of 347 or 360nm (at 360nm the contribution of the covalent adducts is negligible) are shown in figure 2B. Identical rate constants of $k=(3.6\pm0.1)^{-1}min^{-1}$ are obtained, showing that the (+) and (-) enantiomers react with the same rate constants. Using this value of k, solid lines calculated according to eq. (1) are superimposed on the experimentally determined $\Delta A(t)$ points in fig. 2A. We conclude that in the case of the (+) enantiomer a simple model in which intercalated (7) diol epoxide molecules at site I react covalently to give site II adducts, is completely consistent with the kinetic changes in the linear dichroism data.

It is of interest to examine differences in the conformations of the covalent adducts derived from the (+) and the (-) enantiomers. For this purpose, in order to obtain a measurable ΔA signal in the case of adducts derived from the binding of the (-) enantiomer, a highly modified covalent adduct was prepared; this was achieved by increasing the (-)anti-BaPDE concentration in the initial reaction mixture and by utilizing a higher DNA concentration. The absorption and linear dichroism spectra of adducts thus obtained for covalent (+) and (-) BaPDE-DNA adducts are compared in fig. 3. We note that the absorption spectrum of the (-)anti-BaPDE-DNA adduct is broader than that of the (+) adduct, and displays a prominent shoulder at 353nm and a maximum at 348nm. This suggests that the (-) adducts are mixtures of site I and site II adducts, which is confirmed by the ΔA spectrum (fig. 3). While the (+) adduct displays only a positive ΔA type II spectrum which is associated with the dominant N2-deoxyguanine adduct (3,18), the (-) adduct displays both positive and negative ΔA signals. At 355 nm, where the absorbance of site I adducts is dominant, the ΔA signal is negative, while at 345-350 nm, where site II adducts dominate, the ΔA spectrum is positive. A detailed analysis of orientation angles is presented elsewhere (18) for (+) and (-) anti-BaPDE adducts oriented by the pulsed electric field method (12) which, in all respects, gives ΔA spectra similar to those reported here.

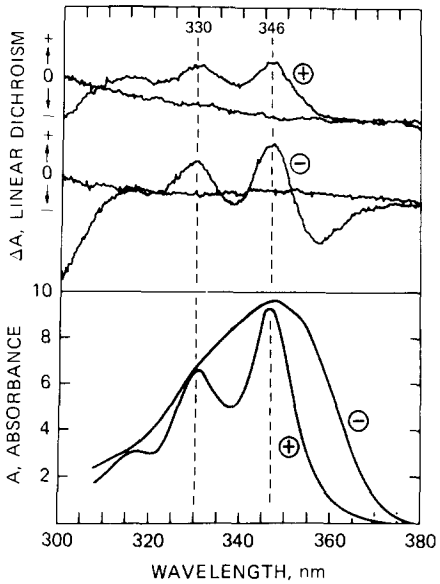

Figure 3. Absorbance and linear dichroism spectra (in relative units) of covalent adducts obtained from the reaction of (+) and (-) anti-BaPDE with DNA (the A and ΔA scales are not comparable for the (+) and (-) adducts). For the (+) adducts: DNA concentration 1.5×10^{-4} M, 1% of bases modified, absorbance at 346nm maximum 0.038. For the (-) adduct: 7.6×10^{-4} M DNA, 1.2% of bases modified, absorbance at 348nm maximum 0.26.

CONCLUSIONS

Both enantiomers form similar intercalative physical complexes and react with the same rates to form tetraols and covalent DNA adducts. The differences in the spatial orientations of the covalently bound diol epoxide residues observed here may be significant in accounting for the prominent differences in the tumorigenic and mutagenic activities of the two enantiomers of anti BaPDE. Furthermore, comparisons between adduct structures (this work,16,18,20) and differences in biological activities of the (+) and (-) enantiomers of anti-BaPDE (3,9), racemic anti and syn BaPDE (21), suggests that the occurence of external site II type of complexes (N2-deoxyguanine adducts), rather than the intercalative type I adducts, are correlated with an enhanced biological activity of this family of stereoisomeric diol epoxides.

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