Location of Excimer-Forming Adducts of (+)-*anti*-Benzo[*a*]pyrene Diol Epoxide in DNA

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Abstract: Covalent adducts of the carcinogenic polycyclic aromatic hydrocarbon (+)-*anti*-benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide ((+)-*anti*-BPDE) in polynucleotides have been studied by fluorescence spectroscopy. The pyrenyl chromophores of the BPDE adducts, linked by the C10 atom to the exocyclic nitrogen of guanine, interact in the photoexcited state, as evidenced by excimer fluorescence. Strong BPDE excimer fluorescence is observed in the alternating poly(dGdC)·poly(dGdC) sequence, whereas it is weak in the homopolymeric poly(dG)·poly(dC) and in calf thymus DNA. No excimer fluorescence is observed for the BPDE adducts in poly(dAdC)·poly(dGdT) or poly(dAdG)·poly(dCdT). It is concluded that the formation of BPDE excimers in polynucleotides requires binding to guanines on different strands on consecutive basepairs. The experimental results are supported by graphics modeling and energy minimization of BPDE adducts in various oligonucleotide sequences. The results show that the most favorable arrangement for excimer formation of the BPDE-dG adducts is in a 5′(dCdG-BPDE)·5′(dCdG-BPDE) sequence, where the pyrenyl chromophores interact in the minor groove.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are widespread environmental pollutants that are produced in combustion processes such as petroleum fuel burning, tobacco smoking, and food preparation at high temperatures.[1,2] A number of PAH are proven carcinogens in animals and are believed to have the same effect also in humans.[3] A general prerequisite for PAH to initiate tumorigenesis is the formation of bay-region diol epoxides and their subsequent covalent binding to specific targets in DNA.[4,5]

Benzo[*a*]pyrene is the most studied PAH and our present understanding of the mechanisms of tumor initiation is largely based on the findings with this compound. Systematic testing for tumorigenic activity of the possible diol epoxide isomers of benzo[*a*]pyrene has clearly shown that benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide [(+)-*anti*-BPDE] is by far the most active form in mammals. The reaction with DNA is stereospecific and sequence specific, predominantly occurring by a trans addition of the exocyclic amino group N2 of guanine to the carbon C10 on the benzylic ring of BPDE.[5,6]

Extensive efforts have been made to characterize the structure of the (+)-*anti*-BPDE–dG adduct in DNA and in polynucleotides using mainly optical spectroscopy techniques.[7,8] Recently solution structures based on NMR results were presented for (+)-*anti*-BPDE and (−)-*anti*-BPDE bound to a DNA oligomer, cf. refs 9 and 10, respectively. In both structures the BPDE, anchored by its C10 to a guanine N2, is located in the minor groove. The adduct of the (+)-enantiomer is directed toward the 5′ end of the modified strand, while the (−)-enantiomer is directed toward the 3′ end. Both enantiomers were found to be stacked with the sugar-phosphate backbone of the partner strand with one face exposed to the solvent. The BPDE adducts were reported to have little effect on the DNA structure; only a slight widening of the minor groove was found.

From molecular mechanics modeling studies it has been found that among several polycyclic hydrocarbon adducts (+)-*trans*-BPDE cause the least helix destabilization.[11] It has also been reported that a 5′-side alignment of the BPDE is the most energetically favorable.[12]

Optical studies of the (+)-*anti*-BPDE–dG adduct structure generally agree with the NMR results. From linear dichroism studies it has been shown that the long axis of the pyrene chromophore is oriented at an angle of less than 35° relative to the average helix axis in polymeric DNA or poly(dGdC)·poly(dGdC).[13–15] It has also been found that the degree of orientation of DNA in flow is lowered by (+)-*anti*-BPDE modification, indicating that the DNA structure is distorted by the adduct.[15]

Further characterization of the properties of the (+)-*anti*-BPDE–DNA adducts has been done by fluorescence spectroscopy. An interesting outcome of these studies was the discovery of excimer fluorescence from the pyrenyl chromophores in the alternating polynucleotide poly(dGdC)·poly(dGdC).[16] Excimer fluorescence was observed only to a considerably lower degree in calf thymus DNA. This discovery immediately suggested that the covalent adduct formation occurs with preference at closely spaced guanines.[17] However, the exact positioning of the in-

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teracting pyrene chromophores could not be determined.

Here we present extended fluorescence studies of (+)-*anti*-BPDE bound to different polynucleotide sequences. We conclude that in order for excimer fluorescence to be observed the two interacting adducts have to be attached to guanines on neighboring basepairs on different strands in the polynucleotide.

The experimental results are supported by graphics modeling and energy minimization of (+)-*anti*-BPDE adducts to N2 of guanine at various sites in different oligonucleotide duplexes.

Materials and Methods

Chemicals. (+)-*trans*-7,8-Dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene ((+)-*anti*-BPDE) was purchased from the National Cancer Institute Carcinogen Reference Standard Repository (Lenexa, KS). The synthetic polynucleotides were purchased from Pharmacia, Uppsala, Sweden. The polynucleotides were dissolved in 5 mM sodium cacodylate buffer, pH 7.0, containing 100 mM NaCl and 1 mM EDTA. The solution was dialyzed against 5 mM sodium cacodylate buffer, pH 7.0, containing 20 mM NaCl several times. The poly(dG)·poly(dC) was first dissolved in 5 mM sodium cacodylate solution at pH 12.0 followed by the same dialysis procedure as the other polynucleotides.

BPDE Modification Procedure. The (+)-*anti*-BPDE–polynucleotide adducts were prepared by adding the (+)-*anti*-BPDE, dissolved in tetrahydrofuran/triethylamine (19:1 v/v), to the polynucleotide solutions. A typical concentration of the stock solution is 1 mg/mL. An appropriate volume of BPDE solution, considering the fraction of BPDE anticipated to react by forming covalent adducts with the polynucleotides,[18] was injected into 4 mL of the 150 μM polynucleotide solution. The mixtures were left overnight at room temperature to ensure complete reaction. Subsequently the incubations were extracted 12 times with diethyl ether to remove the hydrolysis products formed (benzo[*a*]pyrene–tetraols, BPT). The samples were further purified by extensive dialysis at 4 °C against 5 mM sodium cacodylate buffer, pH 7.0, containing 20 mM NaCl. All fluorescence spectra were collected in the presence of 0.2 M acrylamide in order to suppress fluorescence from free BPT.

The degree of modification was spectrophotometrically determined to be between 1.0 and 1.5% for all samples. The extinction coefficients used were ϵ_{346} = 29 500 cm^{-1} M^{-1} for bound BPDE, ϵ_{254} = 8400 cm^{-1} M^{-1} for poly(dGdC)·poly(dGdC), ϵ_{258} = 6500 cm^{-1} M^{-1} for poly(dAdC)·poly(dGdT), ϵ_{253} = 7400 cm^{-1} M^{-1} for poly(dG)·poly(dC), and ϵ_{258} = 6700 cm^{-1} M^{-1} for poly(dAdG)·poly(dCdT).

Instrumentation. The fluorescence spectra were recorded on an Aminco SPF-500 "corrected spectra" spectrofluorimeter. The graphics modeling was done on a Silicon Graphics Personal Iris 4D/25G.

Graphics Modeling and Energy Minimization. The starting coordinates for (+)-*anti*-BPDE were taken from the crystal structure of Neidle et al.[19] and were transformed into Protein Data Bank format. The coordinates for the oligonucleotides were generated as standard B-form DNA with the graphics program Hydra, Iris Release 1.2, Polygen Corporation, obtained from R. Hubbard, University of York, York, England. Docking of the BPDE adduct to the oligonucleotide was done with Hydra. The BPDE-oligonucleotide complex was created with the molecular dynamics program X-PLOR,[20] version 3.0. The length of the BPDE-guanine linking C-N bond is not exactly known. Values ranging from 1.44 to 2.0 Å were tried and the parameter was found not to be critical for this study. The force constant of this bond was set to 350 kcal/mol $Å^{-2}$. For parametrization of the BPDE-molecule, see supplementary material. The resulting BPDE-oligonucleotide adduct was subjected to conjugate gradient energy minimization, performed in 400 steps using the Powell algorithm in the X-PLOR program. The force field used, included the empirical terms for the bond, angle, dihedral angle, improper angle, van der Waals and electrostatic energies.[20,21] In addition, an explicit hydrogen bond term was used to keep the basepairing of the oligonucleotides intact. Effects of the solvent and counterions were approximated by use of a distance dependent dielectric constant in the electrostatic energy potential.

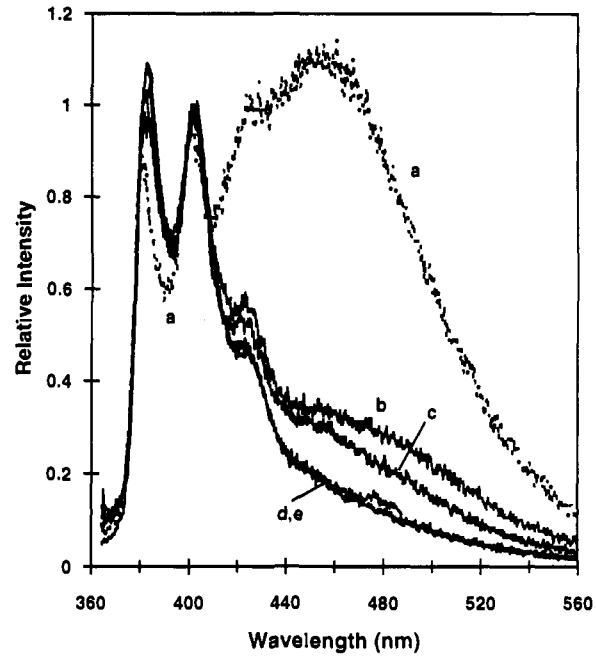

Figure 1. Fluorescence emission spectra of (+)-*anti*-BPDE adducts in poly(dGdC)·poly(dGdC) (a, dotted curve), poly(dG)·poly(dC) (b), calf thymus DNA (c), and poly(dAdC)·poly(dGdT) and poly(dAdG)·poly(dCdT) (d,e). The excitation wavelength was kept at 355 nm. The spectra were collected in the presence of 0.2 M acrylamide in order to suppress fluorescence from free BPT. Each spectrum was normalized to 1.0 at 405 nm.

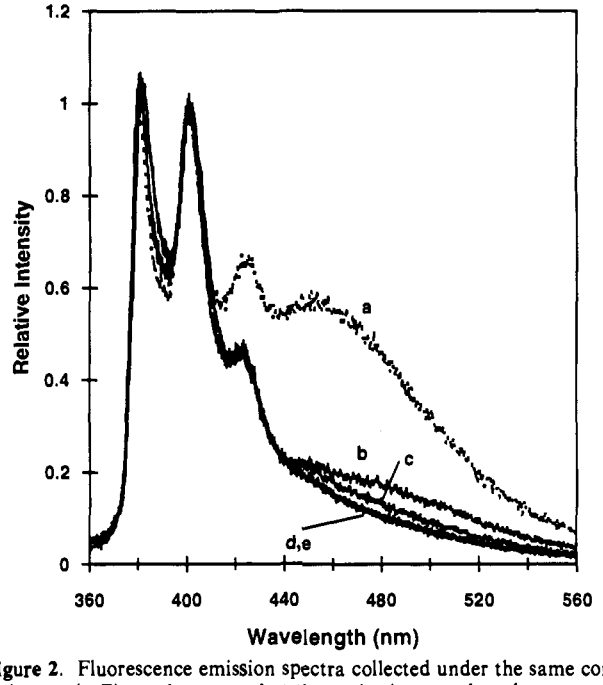

Figure 2. Fluorescence emission spectra collected under the same conditions as in Figure 1, except that the excitation wavelength was set to 345 nm. Each spectrum was normalized to 1.0 at 405 nm.

Results

Fluorescence. Fluorescence emission spectra of (+)-*anti*-BPDE adducts in various polynucleotides are shown in Figure 1. The short wavelength emission band with vibronic structure (365–420 nm) is due to fluorescence from BPDE monomers. The broad unstructured emission band above 440 nm is due to excimer fluorescence from excited-state complexes of interacting benzo[*a*]pyrene adducts. The lack of structure in the shape of the emission peak is due to the fact that the excited state complex is not stable in the ground state and the emission is therefore a transition between poorly defined energy levels. It is clearly seen

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from Figure 1 that the excimer fluorescence is extensive when the BPDE is bound to the alternating sequence poly(dGdC)·poly(dGdC), whereas only weak contributions are seen from the adducts in the homopolymer poly(dG)·poly(dC) or calf thymus DNA. In poly(dAdC)·poly(dGdT) and poly(dAdG)·poly(dCdT) where the guanine bases are positioned on the same strand and spaced by one base, no excimer fluorescence is observed.

In Figure 2 are shown the emission spectra of (+)-*anti*-BPDE adducts in polynucleotides under the same conditions as in Figure 1, the only difference being the excitation wavelength. It is clear that the excimer fluorescence is considerably weaker when exciting at 345 nm as compared to 355 nm. However, the excimer fluorescence is still much more pronounced when BPDE is bound to poly(dGdC)·poly(dGdC) than to the other polynucleotides. The stronger excimer fluorescence observed upon excitation at 355 nm, as compared to 345 nm, reflects the different excitation spectra of the BPDE adducts fluorescing as monomers and the adducts that form excimers.[17]

The fluorescence intensity in the 380–420-nm region is significantly weaker for (+)-*anti*-BPDE adducts in poly(dGdC)·poly(dGdC) as compared to adducts in the other polynucleotides at the same binding ratio (data not shown). The loss of monomer intensity in poly(dGdC)·poly(dGdC) can be explained by the substantial formation of excimers. A rough estimate of the proportion of the BPDE adducts fluorescing as excimers in poly(dGdC)·poly(dGdC) was made as follows. The monomer fluorescence spectrum was calculated by subtracting the excimer contribution in the 360–420-nm region, which was obtained by extrapolation. The resulting monomer intensities at 385 and 405 nm were compared with those of the (+)-*anti*-BPDE adducts in DNA, which has a similar absorption spectrum but only exhibits minor excimer emission. Spectra of samples with a similar BPDE adduct/nucleotide ratio were used for comparison. Subtraction of the excimer contribution resulted in an intensity loss of 59% at 385 nm and 63% at 405 nm, when λ_{ex} = 345 nm. With λ_{ex} set to 353 nm the intensity loss was found to be 61% at 385 nm and 67% at 405 nm. Hence, approximately 60% of the (+)-*anti*-BPDE adducts in poly(dGdC)·poly(dGdC) may be involved in excimer formation at an adduct concentration of 0.01 per nucleotide.

Graphics Modeling. The oligonucleotide sequences below were chosen as models for the polynucleotides poly(dGdC)·poly(dGdC) (sequences **1** and **2**), poly(dG)·poly(dC) (sequence **3**), poly(dAdC)·poly(dGdT) (sequence **4**) and poly(dAdG)·poly(dCdT) (sequence **5**). The structures were created by graphically docking

1

$C_1\ G_2C_3G_4C_5\ G^*_6C_7G_8C_9G_{10}$

$G_{10}C_9G_8C_7G^*_6C_5\ G_4C_3G_2C_1$

2

$C_1\ G_2C_3G^*_4C_5\ G_6C_7G_8C_9G_{10}$

$G_{10}C_9G_8C_7\ G^*_6C_5G_4C_3G_2C_1$

3

$G_1\ G_2G_3G_4G^*_5G^*_6G_7G_8G_9G_{10}$

$C_{10}C_9C_8C_7C_6\ C_5\ C_4C_3C_2C_1$

4

$A_1\ C_2A_3C_4\ A_5C_6\ A_7C_8A_9C_{10}$

$T_{10}G_9T_8G^*_7T_6G^*_5T_4G_3T_2G_1$

5

$A_1\ G_2A_3G^*_4A_5G^*_6A_7G_8A_9G_{10}$

$T_{10}C_9T_8C_7\ T_6C_5\ T_4C_3T_2C_1$

the two (+)-*anti*-BPDE molecules to the oligonucleotides, at the positions indicated by an asterisk. Covalent trans linkages were generated between C10 on the BPDE and N2 on the guanine. Given the stereochemistry of the covalent adducts (the (+)-enantiomer of *anti*-BPDE in the trans position) and keeping the NMR structure of Cosman et al.[9] in mind, the pyrenyl ring system was directed to the 5′ side of the strand to which it is attached. The energies of the structures were minimized to relax strains and to avoid unfavorable van der Waals contacts. In all cases, except with sequence **2**, we found it possible to fit the two BPDE adducts into the minor groove of the oligonucleotides without causing any significant distortions of the DNA structure. In sequence **2** the saturated rings of the BPDE molecules were found to clash in the minor groove and therefore cause considerable distortions of the DNA structure during energy minimization (e.g. base tilt and hydrogen bond breakage).

In order to estimate the extent of the structural changes in the oligonucleotides caused by the BPDE adducts, we measured the root-mean-square deviation (RMSD) between unmodified and modified structures. For all sequences studied, except with sequence **2**, the overall RMSD was found to be smaller than 0.9 Å when comparing the energy minimized structures before and after addition of the two BPDE molecules. The RMSD value measured for any individual base was, with the exception of sequence **2**, found to be less than 1.3 Å.

In sequence **1**, where the adducts are attached to different strands of an alternating dCdG sequence, the overlap between the pyrenyl residues of the two BPDE adducts was found to be extensive in the model, cf. Figure 3a. The separation between the two pyrene molecular planes was found to be less than 4.0 Å, measured as the shortest intermolecular carbon–carbon distance. Since a considerable pyrene–pyrene overlap is required for an excimer to be stable, sequence **1** appears to be a likely model for BPDE excimers in poly(dGdC)·poly(dGdC). In Figure 3b,c,d,e are shown the energy minimized structures of the BPDE adducts in the sequences **2**, **3**, **4**, and **5**, respectively. For these oligonucleotide sequences the overlap between the pyrenyl chromophores appears to be zero or very small, or the separation between the adducts is too large for interaction to take place. This agrees well with the absence of excimer fluorescence from these adducts.

Discussion

This study shows that the formation of excimers between covalent (+)-*anti*-BPDE–dG adducts occurs in poly(dGdC)·poly(dGdC) whereas in other polynucleotide sequences or in natural DNA no or only very weak excimer fluorescence is observed. Here we focus on the structural requirements for excimer formation between BPDE adducts in polynucleotides.

It has been suggested that the fluorescence here ascribed to originate from excimers possibly could be due to exciplexes formed between the pyrenyl chromophore and guanine.[22] The fact that long wavelength fluorescence only appears in one of the studied sequences strongly argues against pyrene–guanine exciplexes. Also the earlier reported observation of a relative increase of the excimer fluorescence with increasing binding ratio is in conflict with the formation of exciplexes.[17]

The alternating dGdC sequence allows the formation of two closely located adducts either on the same strand or on different strands. Were the two adducts situated on the same strand they could either be on neighboring bases or be separated by one residue (cf. sequences **3** and **4**, respectively). Since no excimer fluorescence is observed from the BPDE modified poly(dG)·poly(dC), poly(dAdC)·poly(dGdT), or poly(dAdG)·poly(dCdT) we conclude that the adducts have to be formed on different strands in order to form excimers.

Adduct formation on different strands gives the option of a BPDE having another BPDE either on its 3′ or 5′ side (cf. sequences **1** and **2**, respectively). Sequence **2** corresponds to the case when two adducts are formed on different strands having the other adduct to its 3′ side, on the flanking basepair. Judged by the model structure, Figure 3b, this option seems incompatible with the formation of excimers, because of the large separation of the pyrenyl chromophores. We are thus left with the case where modification has taken place on different strands with one adduct having another adduct to its 5′ side (sequence **1**). From the model structure it should be clear that the adducts have to be attached

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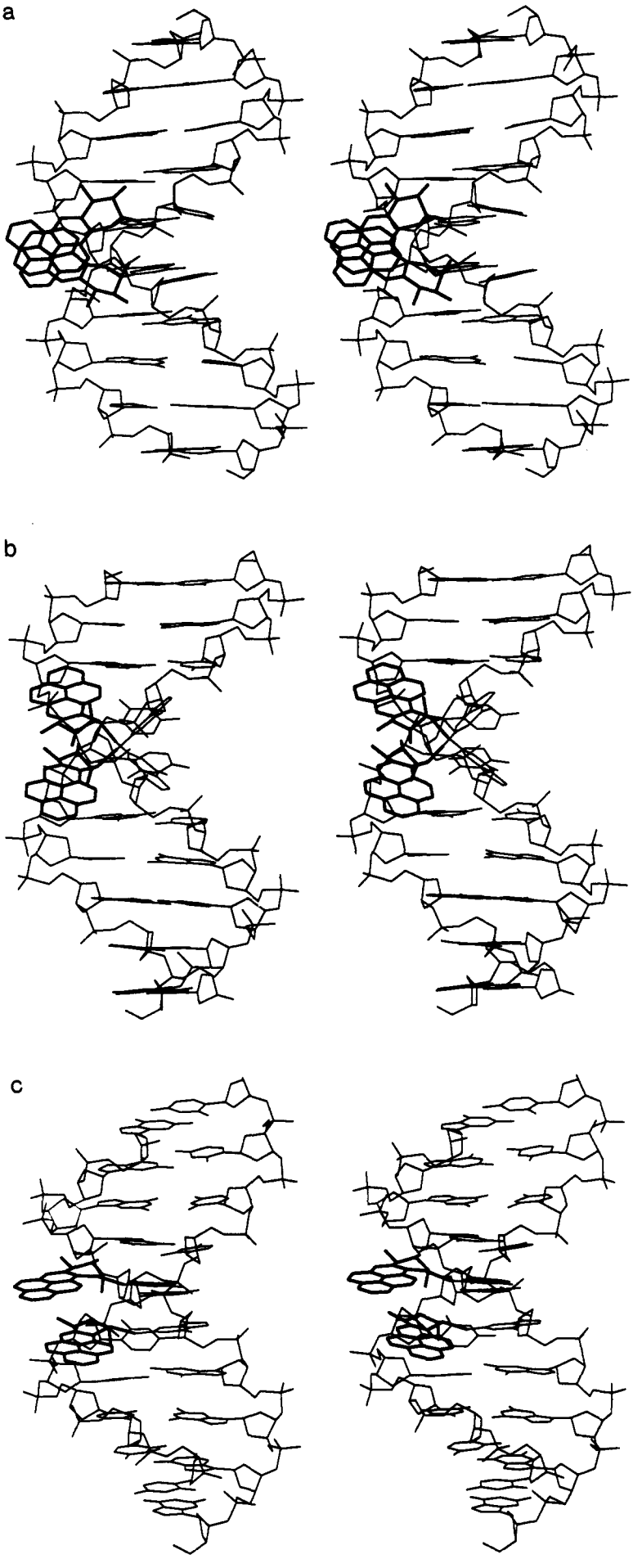
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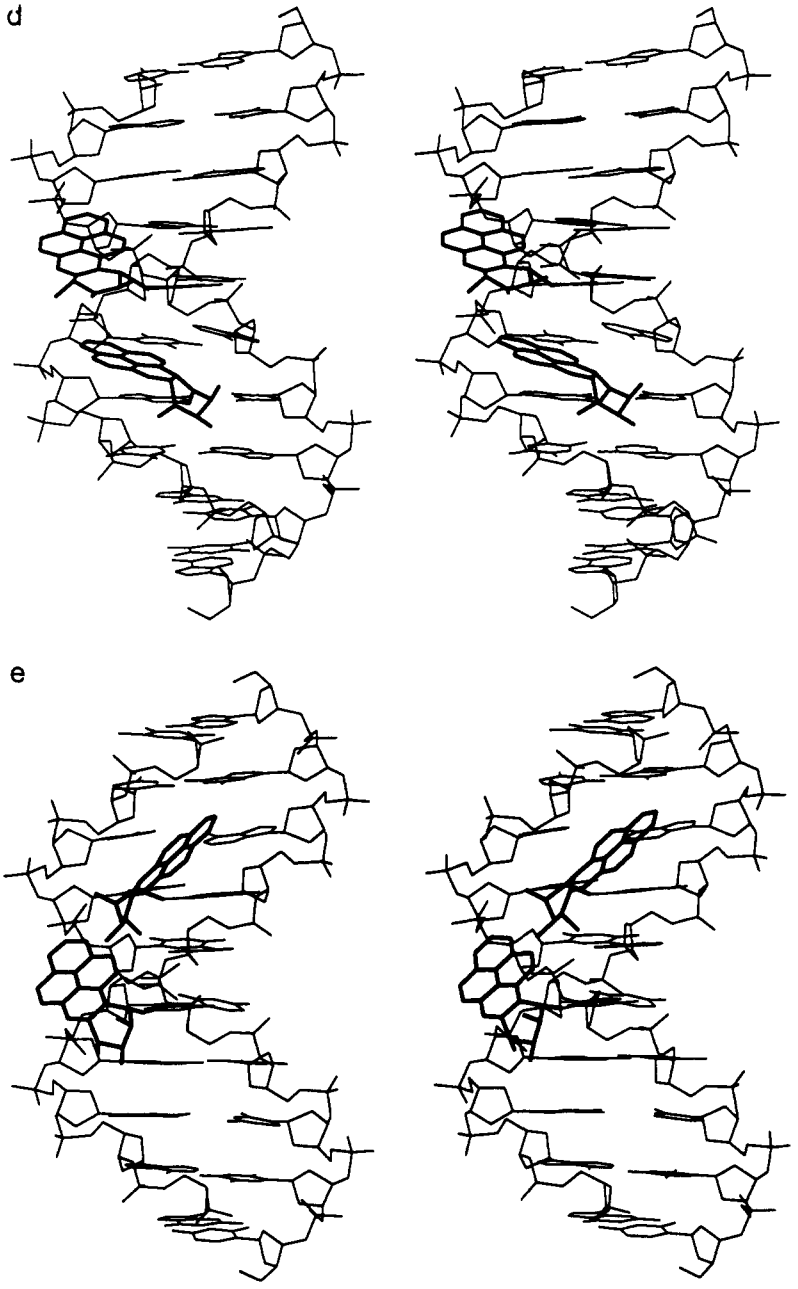

Figure 3. Energy minimized structure of two (+)-*anti*-BPDE adducts in $(dCdG)_5 \cdot (dCdG)_5$, sequence **1** (proposed excimer arrangement) (a), in $(dCdG)_5 \cdot (dCdG)_5$, sequence **2** (b), in $(dG)_{10} \cdot (dC)_{10}$, sequence **3** (c), in $(dAdC)_5 \cdot (dGdT)_5$, sequence **4** (d), and in $(dAdG)_5 \cdot (dCdT)_5$, sequence **5** (e).

to consecutive basepairs to interact. We thus propose this to be the most suitable arrangement for BPDE excimer formation in DNA. This conclusion is compatible with the NMR structure showing that the aromatic part of the (+)-*anti*-BPDE adduct is directed to the 5′ side of the modified dG.[9] Two adducts arranged as in sequence **1**, on consecutive basepairs in an alternating dGdC sequence making contacts with the phosphate–sugar backbone of the partner strand, would make close contacts in the minor groove.

From studying the energy minimized model structures, it is easy to see that the alternating dGdC sequence **1** with the adducts on the two G_6 bases provides the only extensive pyrene–pyrene overlap (cf. Figure 3a). In the other sequences, **2**, **3**, **4**, and **5**, representing the different polynucleotide sequences discussed above, it seems impossible to accomplish a face-to-face arrangement of the two pyrenes, which is required for the formation of excimers (Figure 3b,c,d,e). Thus the modeling experiments are in full agreement with the experimental fluorescence results.

In the modeling experiments only the structural geometries of the excimers were considered. Since the excimer is only stable in the excited state and the BPDE adducts only exist as monomers in the ground state, they have to be able to easily move further apart or change their relative orientation within their binding sites. However, the fluorescence excitation spectra recorded at different emission wavelengths exhibit maxima at different wavelengths.[17] The excitation spectrum observed at λ_{em} = 500 nm (selecting excimer fluorescence) is red shifted by some 5 nm compared to the excitation spectrum observed at λ_{em} = 380 nm (corresponding mainly to the excitation of BPDE adducts fluorescing as monomers). This indicates that the excimer-forming adducts interact also in the ground state. The nature of this interaction is likely to be a partial π–π electronic overlap of the pyrenyl chromophores somewhat lowering their excitation energy. It seems possible that the structural differences between the ground state and the excited state complex are relatively small.

Linear dichroism and NMR experiments have previously shown that the orientation of the pyrenyl long axis of the ground state adduct forms a small angle with the DNA helix axis. By inference from the molecular model in Figure 3a, it seems as if the formation of an excimer requires a similar orientation of the interacting

pyrenyl chromophores. The equilibrium distance between the two molecular planes in an excimer of pyrene free in solution has been reported to be 3.4 Å.[23,24] Another observation supporting the pyrenes being closely located in the ground state is the red shift of the absorption that increases with the amount of adduct in the polynucleotide. The squeezed geometry of two pyrenes, face to face, in the minor groove, cannot possibly allow any extensive separation of the adducts without widening the groove considerably.

The fluorescence polarization anisotropy of the excimer fluorescence has been measured earlier, but it could not easily be interpreted in terms of pyrene motion during its fluorescence lifetime.[17] However, it has been reported that the adducts undergo local motion described by a rotational correlation time of ~0.4 ns which should allow considerable motion to take place during its average fluorescence lifetime (three lifetime components of 0.4, 3, and 24 ns have been measured).[25]

The structures in Figure 3 are intended to illustrate that it is possible to accommodate two pyrene molecules in an excimer-like geometry in the minor groove. The structures have only been subjected to energy minimization without solvent molecules. This is an obvious limitation when modeling this system since it is clear from numerous experiments that the hydrophobicity of the pyrene moiety is of importance for the binding geometries of the BPDE–DNA complexes.[26,27]

The formation of (+)-*anti*-BPDE excimers in polynucleotides is associated with a loss of fluorescence intensity from BPDE monomers. A similar loss of monomer fluorescence intensity has previously been observed for BPDE in cyclodextrin inclusion complexes, where BPDE forms excimers.[28] From a comparison with adducts in DNA, we estimate that approximately 60% of the (+)-*anti*-BPDE adducts in poly(dGdC)·poly(dGdC) may interact to form excimers at an adduct concentration of 0.012 per nucleotide. This has to be considered as an approximate estimation for a couple of reasons. First, the spectral shape of the pure monomer fluorescence of (+)-*anti*-BPDE in poly(dGdC)·poly(dGdC) cannot be obtained. However, at 100 K, at which temperature the excimer fluorescence is considerably weaker, the fluorescence spectra of (+)-*anti*-BPDE adducts in poly(dGdC)·poly(dGdC) and DNA are very similar.[29] Second, the monomer quantum yields may differ for adducts in poly(dGdC)·poly(dGdC) and DNA. It has been shown that the fluorescence lifetimes of (+)-*anti*-BPDE adducts in poly(dGdC)·poly(dGdC) and in DNA are slightly different, although they are within the same range.[25,29,30] The apparent excimer/monomer ratio may be expected to depend on the excitation wavelength. However, the results obtained with λ_{ex} set to 345 and 353 nm do not exhibit a strong such dependence. The excimer/monomer ratio can also be expected to increase as the number of adducts per nucleotide is increased, as has been discussed earlier.[17]

The extensive loss of monomer emission at a relatively small adduct/nucleotide ratio (0.01) is in good agreement with the earlier conclusion that the binding of (+)-*anti*-BPDE to guanine bases in DNA is favored at guanines close to previously modified bases.[17] This may be a new type of recognition, namely ligand–(ligand–DNA) interaction as distinct from ligand–DNA and (ligand dimer)–DNA interactions. Here we have shown that it is possible to fit two adducts into the minor groove. Linear dichroism experiments with (+)-*anti*-BPDE modified poly(dGdC)·poly(dGdC) showed that the degree of DNA orientation decreases as the degree of BPDE modification is increased.[15] This may be an effect of highly modified regions in which DNA structure distortions are accumulated to result in DNA helix axis curvature or flexibility. Thus this new recognition mechanism may be of importance for polynucleotide structure and/or dynamics and thereby possibly also of importance for recognition of repair enzymes.

So far no biological significance has been related to the formation of excimers between BPDE adducts in DNA. Since the (+)-*anti*-BPDE–dG adducts are dominated by trans adducts of type II, the adducts having the potency of forming excimers in their photoexcited states most likely are of type II.[31,32] Furthermore, it has been shown that the trans N2–dG adducts in poly(dGdC)·poly(dGdC), as compared to other polynucleotides, are the ones structurally most similar to the adducts formed in DNA.[15] These adducts also seem to be responsible for the GC → TA transversion mutations observed in human cells after treatment with racemic *anti*-BPDE.[33] Earlier results have suggested that the BPDE adducts are more likely to be formed close to each other, rather than being randomly distributed along the polynucleotide.[17] It has also been suggested that the adduct distribution between different types of adducts, as judged by their excited-state fluorescence properties, changes with the degree of modification.[31,32] Thus it seems probable that there are physical interactions between BPDE molecules prior to the formation of covalent bonds to dG in the polynucleotide.

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Supplementary Material Available: Parametrization details of (+)-*anti*-BPDE (1 page). Ordering information is given on any current masthead page.

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