These comments apply to molecules in both vapor and solution phases. However, owing to the solvent cage effect, in solution many collisions occur between oxygen and energy donor molecules during each encounter. This statistically increases the probability for energy transfer, compensates for the weaker coupling between ψ_i and ψ_{CT}, and makes the quenching constants comparable for all carbonyl and dicarbonyl compounds.

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Physical Binding of Tetraols Derived from 7,8-Dihydroxy-9,10-epoxybenzo[*a*]pyrene to DNA

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Abstract: The major reactive metabolite of the carcinogen *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-BPDE) either reacts covalently with DNA or is hydrolyzed to the tetraol 7,8,9,10-tetrahydroxytetrahydrobenzo[*a*]pyrene (BPT). In this work it is shown that BPT binds noncovalently to DNA in aqueous buffer solution (5 mM sodium cacodylate buffer, pH 7.1) at 25 °C. This binding, at values of the binding ratio $r \approx 10^{-3}$, defined as the ratio of bound BPT molecules per DNA base, is characterized by two types of binding sites. Site I is characterized by a 10-nm red shift in the absorption spectrum (a shift from 343 to 353 nm for the most intense absorption band of BPT), a complete quenching of the fluorescence of BPT at this site, and a negative linear dichroism spectrum. These properties are characteristic of an intercalation-type complex, in which the BPT molecule is sandwiched between adjacent base pairs of DNA. Equilibrium dialysis and absorption and fluorescence spectroscopy are the techniques utilized to demonstrate that there is a second type of binding site (II). This binding site is characterized by the following: (1) no shift in the absorption spectrum with respect to that of free BPT molecules in the buffer solution; (2) unchanged fluorescence yield, decay time, and susceptibility to oxygen quenching. It is proposed that site II corresponds to an external type of binding site of BPT on the DNA molecule, and the similarity between this type of binding and that of the covalent adduct formed between *anti*-BPDE and DNA is noted. At the low values of r studied here ($^1/_{950}$–$^1/_{4700}$), the ratio of BPT molecules at sites I and II lies in the range of 2–4, while 16–38% of the total BPT molecules initially added remain free in solution.

Introduction

Carcinogenic polycyclic aromatic hydrocarbons (PAH) have been demonstrated to undergo metabolic conversion[1] to reactive diol epoxide intermediates which bind covalently to nucleic acids in living cells. Benzo[*a*]pyrene, a ubiquitous environmental pollutant, has been most extensively studied, and *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-BPDE) has been strongly implicated by several lines of evidence as it ultimate carcinogenic form.[2-7] In particular, *anti*-BPDE has been identified as the principal metabolite of benzo[*a*]pyrene which binds to DNA in rodent, bovine, and human tissue. Binding occurs preferentially to guanine[7-9] and to a minor extent to adenine and to cytosine.[10]

The reaction of *anti*-BPDE with DNA can be conveniently carried out in the laboratory,[9-12] and sufficient quantities of the complexes can be prepared for their physicochemical and biochemical characterization.[13-19]

In aqueous solutions, *anti*-BPDE hydrolyzes to cis and trans tetraols (7,8,9,10-tetrahydroxytetrahydrobenzo[*a*]pyrene), a reaction which has been studied extensively.[20-23] The rate of decomposition of *anti*-BPDE to the benzo[*a*]pyrene tetraols (BPT) depends on the pH and on the other components present in the aqueous solution. In an effort to gain a better understanding of the mechanism of reaction between *anti*-BPDE and DNA, we have recently studied the kinetics of this reaction in aqueous solution at 25 °C utilizing absorption, fluorescence, and electric linear dichroism spectroscopic methods.[24] It was found that the hy-

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drolysis of *anti*-BPDE to its tetraols is markedly accelerated in the presence of DNA when the initial DNA phosphate/*anti*-BPDE ratio is 50 or more and that the *anti*-BPDE is completely reacted less than 5 min after mixing (pH 7.1, 25 °C in 5 mM sodium cacodylate buffer solution). In the absence of DNA, this hydrolysis is complete after 90 min under the same conditions. Since the tetraols also bind to DNA, this noncovalent interaction obscures the covalent binding of *anti*-BPDE to DNA.

In an effort to separate the effects of these competing covalent and noncovalent interactions with DNA, we have studied the physical binding to DNA of the chemically unreactive tetraols derived from the prior hydrolysis of *anti*-BPDE. Because these tetraols are stable, their physical binding to DNA can be studied conveniently by utilizing such techniques as equilibrium dialysis. In this paper a combination of fluorescence, absorption, and electric linear dichroism techniques is utilized to show that there are two major types of noncovalent binding sites of the tetraol to DNA. Site I is an intercalation-type binding site, while site II displays most of the fluorescence and absorption properties of the covalent *anti*-BPDE–DNA adduct[17,18] and is probably an external binding site.

Experimental Section

Materials. The tetraol 7,8,9,10-tetrahydroxytetrahydrobenzo[*a*]pyrene (BPT) was prepared by hydrolysis of *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene in an acidified water–methanol (2:1) mixture. The methanol was removed by flushing with nitrogen. High-molecular-weight calf thymus DNA was obtained from Worthington Biochemicals (Freehold, N.J.) and was dissolved in 5 mM sodium cacodylate (pH 7.1) buffer solution. The BPT–DNA complexes were prepared by adding appropriate amounts of BPT to the DNA solutions.

Absorbance measurements were performed on a Cary 14 spectrophotometer. Fluorescence emission and excitation spectra were determined utilizing a Hitachi MPF2A spectrophotometer.

The fluorescence decay profiles were determined with a single photon counting apparatus which is described in more detail elsewhere.[25] A 330-nm interference filter was placed between the pulsed light source and the sample (contained in a 1 × 1 cm cuvette), while the fluorescence was isolated by means of Corning 3-74 (cutoff wavelength 360 nm) and 405 nm interference filters. Despite the orthogonality of these filters, some scattered light was nevertheless detected when blank DNA solutions (no BPT present) were placed in the apparatus. This scattered light was sometimes comparable to the BPT fluorescence in the first few nanoseconds of the decay curve, particularly when dilute solutions of BPT were used. In such cases the blank DNA and BPT–DNA samples (same concentration of DNA in both cases) were counted for the same length of time, and the blank counts were subtracted from the sample counts before analyzing the decay curves.

The orientation of the BPT molecules complexed to DNA was studied by means of an electric linear dichroism technique. In this technique a voltage pulse of 500 V is applied across two parallel flat platinum plates spaced 3 mm apart and immersed in the DNA solution. The duration of this pulse is typically 2 ms and the DNA molecules are partially oriented during that time. The absorbance of the oriented sample is measured using a piezoelastic modulator which provides a light beam alternately (100 kHz) polarized either parallel or perpendicular with respect to the direction of the applied electric field. The linear dichroism is expressed as $\Delta A = A_{\parallel} - A_{\perp}$, where $A_{\parallel}$ and $A_{\perp}$ are the absorbances measured with the light polarized parallel and perpendicular to the applied electric field, respectively. The ΔA values determined in the wavelength region below 300 nm are negative, since the DNA bases absorbing in this wavelength range tend to orient with their planes perpendicular to the electric field.[26] Flat polycyclic aromatic molecules which bind to DNA by intercalation also display negative ΔA values in the wavelength region corresponding to their absorption spectra in the near-ultraviolet or visible range. Additional details of the apparatus used[16] and the theory of linear dichroism[26] may be found elsewhere.

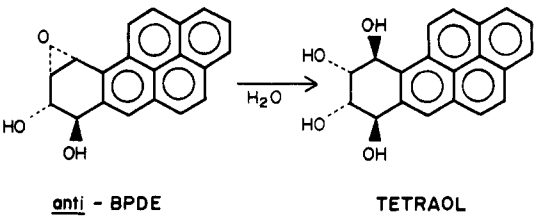

Figure 1. Structure of *anti*-BPDE and its tetraol (BPT) hydrolysis product (the trans stereoisomer is shown).

Equilibrium dialysis measurements were performed by placing a DNA solution into dialysis tubing (no. 250-9u, Sigma Chemical Co., St. Louis, Mo.); this tubing was pretreated as described by McPhie.[27] The DNA solution in the dialysis tube was placed within a flask containing the buffer solution. The tetraol was added either to the inside of the dialysis bag or to the outside of the bag. The dialysis was carried out at 4 °C with gentle stirring. Periodically, portions of the dialysate were tested by measuring the absorbance due to the tetraols (at 343 nm) in order to determine whether equilibrium had been achieved. Equilibrium was usually achieved after 3 days. The same results were obtained when an identical number of moles of the tetraols were placed either within or outside of the dialysis bag at the start of the experiment. It was verified that there was no leakage of the DNA from the bag into the outside solution by measuring the absorbance of the dialysate at 260 nm without any BPT present. The amount of BPT absorbed by the dialysis bag was determined in control experiments in which no DNA was present. All of the dialysis data reported have been corrected for the small amount of BPT adsorbed on the bag.

Results and Discussion

Noncovalent Nature of Tetraol–DNA Complexes. When BPT is allowed to interact with DNA in an aqueous buffer solution, its absorption spectrum changes dramatically and its fluorescence yield decreases. The BPT–DNA complex formed can be easily destroyed by extraction of the BPT from the aqueous phase with an organic solvent. Thus, ten successive extractions with ether lead to a removal of over 95% of the BPT in the aqueous phase; subsequent precipitation of the DNA with ethanol removes the remaining traces of the BPT. This is evidenced by absorption and fluorescence spectroscopy when the DNA is redissolved in aqueous solution. If a covalent complex, derived from the reaction of *anti*-BPDE with DNA, is subjected to the same extraction procedure, a residual absorption remains which cannot be removed by ether extraction and ethanol precipitation. The physical nature of the binding of BPT to DNA described here is, thus, quite different from the covalent binding of BPDE to DNA.

Absorption Studies. The absorption bands of free BPT in buffer solution occur at 313, 327, and 343–344 nm. When DNA is added, a new shoulder, or maximum, appears at 353 nm. The spectrum changes and the 353-nm band becomes more prominent as the concentration of DNA is increased (Figure 2).

The spectra in Figure 2B,C appear to be a superposition of two absorbing species. One of these, corresponding to a red-shifted absorption maximum at 353 nm, we attribute to an intercalation-type complex. The second absorbing species displays a structure at the same wavelength as the free tetraol in Figure 1.

Electric Linear Dichroism (ELD). The orientation of BPT bound to DNA was investigated by the electric linear dichroism technique. An absorption spectrum of a tetraol–DNA solution

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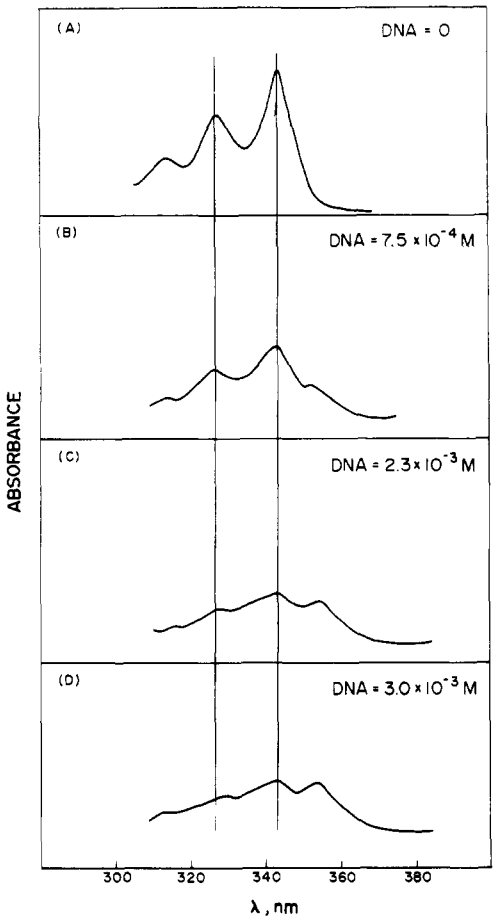

Figure 2. Absorption spectra of a 2.4 × 10^{-6} M BPT solution with different amounts of DNA added (expressed in molarity of phosphate units) in 5 mM sodium cacodylate solution (pH 7.1). (Absorbance scale in relative units.)

and the corresponding ELD spectrum are shown in Figure 3. Under certain conditions the ELD spectrum, defined by the quantity $\Delta A = A_{\parallel} - A_{\perp}$ (see Experimental Section), is proportional to the absorption, A, of the oriented species. This is the case, for example, when there is only one oriented species and one unoriented species with overlapping absorption spectra; the wavelength dependence of ΔA will then resemble the absorption spectrum of the oriented species only. If there are several oriented species with different angles of orientation and with overlapping absorption spectra, the ΔA spectrum may be quite complicated and difficult to interpret.[16]

Fortunately, the ELD spectrum in Figure 3B appears to be simple. It resembles an inverted absorption spectrum of free BPT shifted by about 9–10 nm to the red. Absorption bands corresponding to the unbound form at 314, 327, and 344 nm do not appear. The sign of ΔA is negative. By quantitatively comparing the magnitude of the reduced linear dichroism $\Delta A/A$ below 300 nm with $\Delta A/A$ obtained at 353 nm (approximately equal in sign and in magnitude), utilizing methods outlined elsewhere,[16] it is found that the angle $\theta \geq 85°$. This angle θ defines the relative directions of the axis of the DNA helix and the transition moment of BPT (which lies along the long axis of the pyrene chromophore). Assuming that the planes of the DNA bases themselves are oriented at a 90° angle with respect to the axis of the DNA helix, an intercalated pyrene-like chromophore is characterized by an angle of $\theta = 90°$, since the transition moments below 300 and at 353 nm have the same $\Delta A/A$ values in both sign and magnitude. (Hogan et al.[28] have recently proposed that the tilt of the DNA bases with respect to the axis of the helix is 73°; even if this is the case, the basic conclusions regarding intercalation remain unchanged, even though the calculated value of θ would be different[16] from 90°.)

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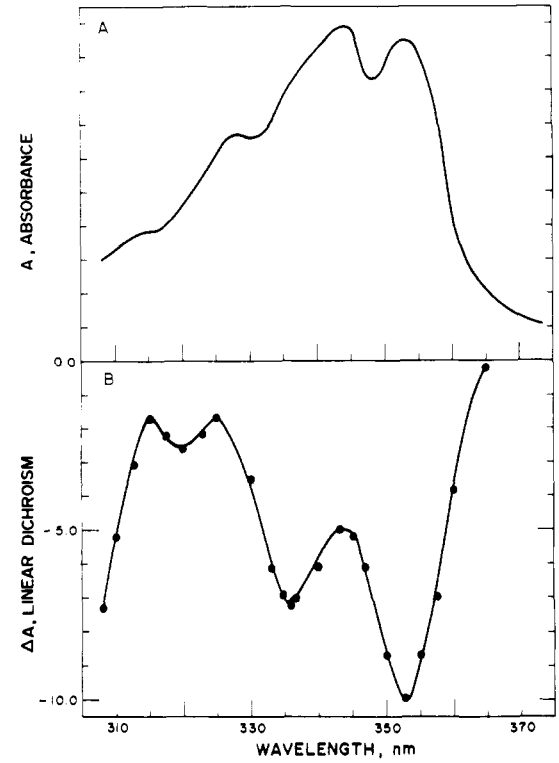

Figure 3. Absorbance and linear dichroism spectra of a 2.1 × 10^{-6} M BPT and 2.7 × 10^{-3} M DNA solution. The reduced linear dichroism values $\Delta A' = \Delta A/A$ at 353 nm relative to this value for the DNA at 260 nm is = 0.99. This quantity is equal to $1 - 3\cos^2\theta$ (ref 16), where θ is the angle between the in-plane electronic transition moment of the pyrene-like chromophore of BPT relative to the orientation of the axis of the DNA helix, calculated to be ≥85°.

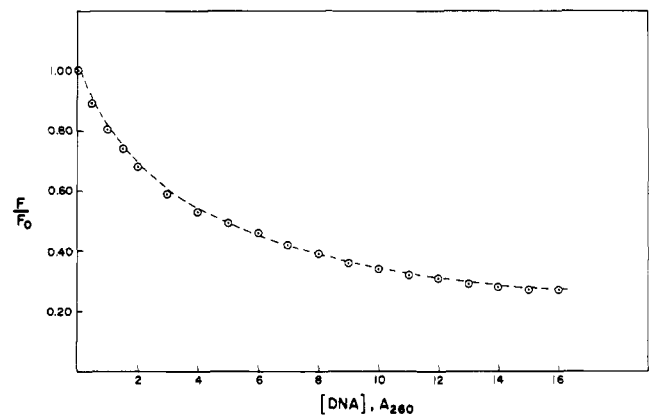

Figure 4. Relative fluorescence yield of a 1.7 × 10^{-6} M BPT solution as a function of DNA concentration expressed in A_{260} units (one A_{260} unit = 1.5 × 10^{-4} M in DNA phosphate units).

These results, a large red absorption shift upon binding to DNA, a negative ΔA spectrum with $\theta \geq 85°$, and strong fluorescence quenching (shown below), are consistent with an intercalation structure. Similar results are observed with the aromatic hydrocarbons pyrene[25] and benzo[*a*]pyrene.[16,25] This type of intercalation binding site will be called site I.

Fluorescence. Upon addition of DNA to a buffer solution containing dissolved BPT, the fluorescence gradually decreases as more DNA is added (Figure 4). In order to ascertain the nature of the fluorescence-emitting species, the fluorescence emission and excitation spectra were determined (Figure 5). The fluorescence excitation maxima (which normally reflect the absorption maxima of the fluorescence-emitting species) correspond to those of the unbound tetraol, which in turn correspond to the

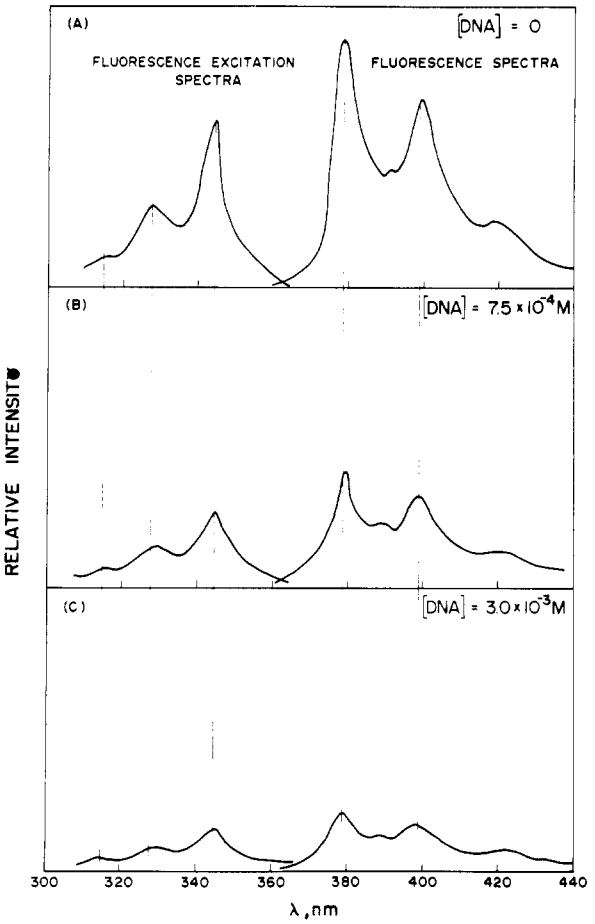

Figure 5. Fluorescence excitation spectra and fluorescence emission spectra of a 2.4×10^{-6} M BPT solution at different DNA concentrations (the intensity units on the vertical scale in A and C are relative to one another).

Table I. Fluorescence Lifetimes (ns) of BPT in the Absence and Presence of DNA (5 mM Cacodylate Buffer, pH = 7.1, 25 °C, [BPT] = 7.0×10^{-6} M)

solution condition	(DNA) = 0	(DNA) = 6.1 A_{260} units
oxygen free	200 ± 3	199 ± 3
air saturated	130 ± 2	128 ± 2

absorption maxima of BPT in aqueous buffer solution without any DNA added (Figure 2). These fluorescence emission and excitation maxima do not change with increasing DNA concentration (Figure 5). We thus conclude that the BPT molecules bound to DNA by intercalation and characterized by the new absorption band at 353 nm have a negligible fluorescence yield. Analogous results have also been observed with pyrene itself.[25]

The fluorescence which is observed with BPT–DNA complexes may be due both to free tetraol and to tetraol bound to DNA at a second site from which fluorescence can occur. If such a bound species has a fluorescence yield and decay time different from that of free tetraol, two components might be observable in the fluorescence decay profile. Such a fluorescence decay curve obtained with the single photon counting technique is shown in Figure 6. The decay is exponential with a minor nonexponential component evident within the first few channels; this minor component, upon more detailed analysis, cannot account for more than 6–7% of the total number of photons emitted. Thus, 92–94% of the fluorescence is attributable to a single component. It is shown in Table I that the decay time observed with the BPT–DNA solution is the same as that of the free tetraol in buffer solution without any DNA added. Molecular oxygen quenches the fluorescence of both the BPT–DNA solution and free BPT with the same efficiency. The Stern–Volmer quenching constant[25]

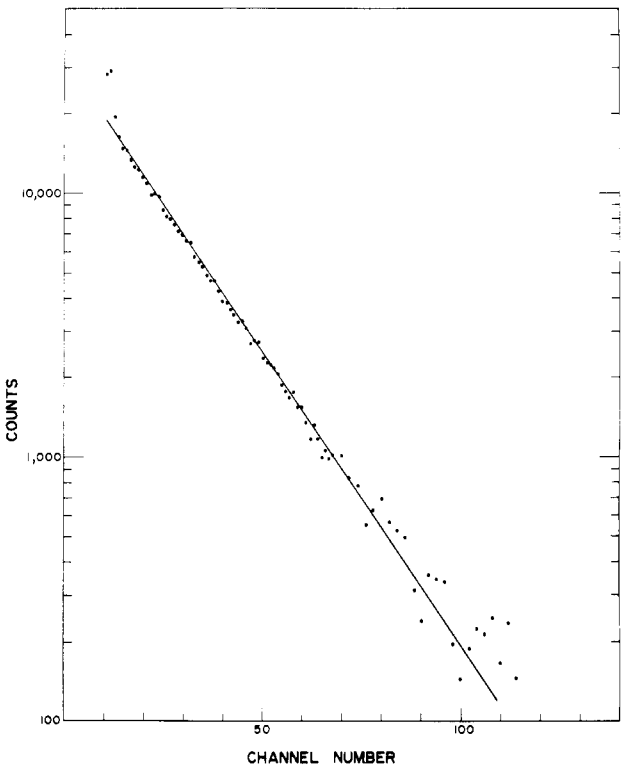

Figure 6. Fluorescence decay profile of a BPT–DNA solution obtained by a single photon counting technique. Time scale 6.86 ns/channel. [DNA]/[BPT] = 1300, BPT concentration is 5.9×10^{-7} M, air-saturated solution.

calculated from the data in Table I is $\sim 10^{10}$ M^{-1} s^{-1}. This constant is a measure of the accessibility of the polycyclic molecules to dissolved oxygen molecules. The value obtained is characteristic of free polycyclic molecules dissolved in aqueous solution. For aromatic molecules bound to DNA by an intercalation mechanism, this quenching constant is expected to be reduced by a factor of 10–20 with respect to its value in free solution, because of the diminished accessibility of the intercalated chromophores to oxygen.[25,29]

These observations, however, are insufficient grounds for eliminating the possibility that a second DNA-bound fluorescence-emitting species exists, which has the same fluorescence and absorption characteristics as free BPT. In fact, the presence of such a fluorescent DNA-bound species of tetraol is demonstrated below.

Equilibrium Dialysis. The dialysis of a solution of BPT and DNA in buffer solution against a buffer solution is useful for determining the contribution of unbound tetraol to both the absorbance and fluorescence properties of a BPT–DNA solution. The absorption spectra of the contents of the dialysis bag containing the DNA and of the outside solution containing free tetraol only are shown in Figure 7. Due to binding of the tetraol to DNA, the concentration of BPT is considerably greater inside the bag than outside of the bag. Since the concentrations of the *free* tetraol are the same both inside and outside of the bag at equilibrium, a comparison of the fluorescence yields inside and outside of the bag should give the relative concentrations of fluorescent species. For the particular example of Figure 7 (see also Table II), the fluorescence ratio inside/outside of the bag was

$$\frac{F_{in}}{F_{out}} = 1.86 \pm 0.02 \qquad (1)$$

The optical densities at which the relative fluorescence yields were determined were significantly below 0.1 at the excitation wavelength of 343 nm. Furthermore, the excitation spectra of the solution containing the complex and free tetraol are the same

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Table II. Some Results of Equilibrium Dialysis Measurements (for Methods of Analysis See Text and Figure 7)

[DNA], A_{260} units	initial ratios[a] [DNA]/[BPT]	BPT bound[b] [DNA]/[BPT]	[site I][c]/ [site II]	% BPT site I	% BPT site II	% BPT free[d]
11.6	170	953	2.3	43	19	38 (0.39×10^{-5})
15.0	710	2230	2.9	57	20	23 (0.73×10^{-6})
29.0	1000	4740	3.4	65	19	16 (0.48×10^{6})

[a] Before dialysis. [b] At equilibrium. [c] Site I: intercalated BPT; site II: fluorescent complex, probably an outside binding site. $20A_{260}$ units = 3.0×10^{-3} M DNA-phosphorous; the concentration of DNA was calculated on this basis. [d] The molar concentrations of free (unbound) BPT is given in the parentheses.

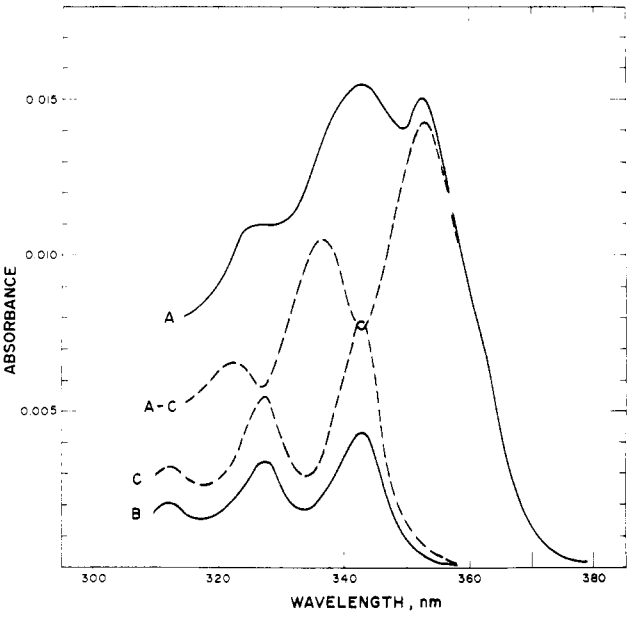

Figure 7. Typical results of equilibrium dialysis experiments. Initially the relative concentration [DNA phosphate]/[BPT] = 1000 inside the dialysis bag (see Table II). Curve A: absorbance of contents of dialysis bag; curve B: absorbance of solution outside of the bag after an equilibration time of 3 days. The fluorescence yield inside the bag was 1.86 times greater than for the solution outside of the bag (343-nm excitation). Curve C: 1.86 × curve B, assumed to be the total absorbance of free BPT molecules plus site II DNA-bound BPT molecules. Curve A–C: difference between curves A and C, assumed to be the absorbance spectrum of BPT molecules at type I site (see text).

(Figure 5), and only a single fluorescence decay component is observed. Under these conditions, the relative fluorescence yield in eq 1 is proportional to the relative concentrations of fluorescence emitting molecules inside and outside of the dialysis bag.

The excess of fluorescence inside the bag over that outside of the bag can thus be attributed to tetraol bound to DNA. For this type of binding site the interactions between the bound tetraol and the DNA macromolecule must be relatively weak, since the absorption and fluorescence properties of the bound tetraol do not appear to be perturbed to any significant extent as compared to these properties displayed by free tetraols. The absorption and fluorescence properties of tetraol at this type of binding site (site II) are very similar to those observed for the adduct obtained upon covalent binding of *anti*-BPDE to DNA; in the latter case, the absorption spectrum is shifted by 1–2 nm at most upon covalent binding, while the fluorescence yield and decay time at a low concentration of DNA, when self-aggregation effects are unimportant, is the same as that of the free tetraol.[17,18] It was also shown that in the covalent adduct the pyrene moiety was located at a site exterior to the DNA double helix.[16] Because of these similarities, we propose that this second binding site of the tetraol to DNA (site II) is also an external binding site.

By utilizing eq 1, the summed absorption spectrum due to (free tetraol) + (site II tetraol) can be estimated. This is done by multiplying the free tetraol absorption spectrum in Figure 7 by the factor of 1.86. The resulting spectrum is also shown in Figure 7 and is subtracted from the total absorption spectrum to yield the spectrum of site I, the intercalated species. The spectrum of site I thus calculated is also shown in Figure 7; the peaks are at 322, 366, and 353 nm. The last two maxima are in excellent agreement with the structure obtained in the linear dichroism spectrum shown in Figure 3. The latter does resemble the inverted absorption spectrum of site I. The relatively good agreement between the dialysis and ELD results reinforces the previously stated conclusion that site I is an intercalation-type nonfluorescent binding site of tetraol.

Site II does not appear to contribute to the ELD spectrum in any obvious manner. First, its relative concentration, as may be seen in Figure 7 and Table II, is lower than that of site I. Furthermore, $\Delta A = \Delta A(\mathrm{I}) + \Delta A(\mathrm{II})$, and each term is proportional to $(3 \cos^2 \theta - 1)$. For $\theta \approx 55°$, this function goes to zero and is small for angles near this value. If the outside bound tetraol (site II) is characterized by an angle around 55°, its contribution to the ELD spectrum is expected to be small. However, the ELD measurements cannot affirm such a hypothesis. Thus, no firm conclusions regarding the orientation of BPT at site II can be reached.

Experiments with the Cis and the Trans Tetraols of BPT. Hydrolysis of *anti*-BPDE in aqueous solution gives rise to a cis and a trans isomer of BPT. The trans isomer (the two OH groups formed at the site of the oxirane oxygen are on opposite sides of the plane of the molecule) is depicted in Figure 1 and is the major product (~60–90%) of the hydrolysis of *anti*-BPDE.[21,22,30] In this work we have utilized stereoisomeric mixtures of cis and trans tetraols. However, utilizing the pure cis and trans (separated by high-pressure liquid chromatography) we have observed that their properties, either in buffer solution with DNA or in the presence of DNA, were the same, within experimental error, as those observed with the stereoisomeric mixture. The fluorescence and absorption spectra, fluorescence decay times, and binding to DNA as monitored by these techniques were the same for stereoisomeric mixtures and the separate stereoisomers.

Binding Constants. From the point of view of eventually gaining insight into the reaction mechanisms which occur in vivo, it appears important to investigate the reaction between *anti*-BPDE and DNA when the latter is present in excess; thus, the physical binding of tetraols was also studied at relatively low concentrations of BPT. In the experiments reported here, the binding ratio r = BPT/DNA phosphate is of the order of 10^{-3}. Utilizing data of the type shown in Table II and the standard equilibrium expression $k = r/C_0$ (where C_0 is the concentration of free BPT in solution), binding constants for sites I and II can be estimated. Values of $K_{\mathrm{I}} \approx 1000\ \mathrm{M}^{-1}$ and $K_{\mathrm{II}} \approx 400\ \mathrm{M}^{-1}$ are obtained. It has been shown[31] that more accurate values of K can be obtained only by performing measurements over a wider range of r, namely, in the range of $0.2 < r < 0.8$. A more complete investigation of the binding isotherm would thus be necessary.

Conclusions

The equilibrium dialysis results shown in Table II indicate that even at high molar ratios of DNA-phosphorous/tetraol a considerable fraction of the tetraol remains free in solution. This is due to the enhanced water solubility of BPT because of the presence of the four OH groups. The physical binding of the tetraols by intercalation, however, accounts for the major fraction

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of the tetraol in DNA solution at high DNA concentrations. Binding by intercalation is due to a "hydrophobic" interaction between the π electrons of the pyrene moiety of BPT and those of the DNA bases. In contrast, the physiochemical properties of the second type of binding site (II) are such that the interaction between the pyrene chromophore and DNA are weaker. The fluorescence yield, decay profile, and absorption spectra appear to be the same as those of unbound tetraol and similar to the same properties displayed by the covalently bound adduct obtained by reacting *anti*-BPDE with DNA. Electric linear dichroism results are consistent with an external binding site for the covalent adduct.[16]

These results indicate that *anti*-BPDE may also bind physically to DNA in a manner similar to BPT, before reacting covalently with DNA. If this is the case, then it appears that such covalent interactions are favored at site II.

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Functional Design of Heme Proteins: Reaction with Linear Ligands

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Abstract: The reactivities of heme proteins are compared with those of an octapeptide model system derived from cytochrome *c*. Comprehensive studies of the equilibria and kinetics of ligation of linear (CN^-) and bent (N_3^-) ligands are reported for myoglobin and the model. The equilibrium constants are 2.7×10^8 M^{-1} for MbCN, 1.5×10^6 M^{-1} for OPCN, 2.5×10^4 M^{-1} for MbN_3, and 27.6 M^{-1} for OPN_3. The forward rate constants are 2.3×10^4 M^{-1} s^{-1} for MbCN and 1.4×10^6 M^{-1} s^{-1} for OPCN. Reverse rate constants, calculated from the equilibrium constants and forward rate constants, are 8.4×10^{-5} s^{-1} for MbCN and 0.95 s^{-1} for OPCN. These data provide estimates of the relative importance of protein-ligand interactions in modifying ligand binding. Estimates are made of the relative importance of electrostatic effects, steric effects, and differential M-L bond strengths. Contrary to previous suggestions, steric effects appear to be energetically small in these systems, while electrostatic effects dominate. Ligation kinetics appear to be dominated by conformational effects.

Introduction

The reactions of heme proteins with small ligands have been studied extensively to gain insight into the nature of the binding site. Nevertheless, the factors which govern the reactivity of heme proteins are not entirely understood. In the unliganded form of myoglobin the entrance to the heme pocket is too small for a ligand to have direct access to the binding site. The residues which block the opening are thought to swing out of the way so the ligand may approach the coordination site.[1] Once inside the protein, the ligand may encounter additional obstacles, such as the distal residues, depending on the size and geometry of the ligand.

One approach to understanding the functional design of heme proteins is provided by comparing the chemistry of similar model systems. By comparing the affinity of various ligands for the protein to a model system, the effect of the protein on reactivity can be analyzed. Numerous investigators have used model systems to deduce the nature of oxygen and carbon monoxide binding in myoglobin and hemoglobin, as summarized in recent reviews.[2] These studies indicate the affinity for carbon monoxide in the sterically unhindered model systems is significantly greater than in myoglobin. In MbCO, X-ray studies show the CO is bent or tilted due to steric interactions with the distal residues of the protein surrounding the binding site.[3] The model systems, which have unencumbered axial ligand binding sites, coordinate CO in the expected linear fashion.[4,5] Caughey postulated that heme proteins have lower affinities for CO due to steric effects.[6] There seems to be mounting evidence for this hypothesis from studies with a variety of heme proteins and synthetic model systems, some of which mimic these steric interactions.[6-12] However, the magnitude of these steric effects remains uncertain; some models have CO affinities which approach that of Hb.[10]

We have carried out comprehensive ligand binding studies with the isoelectronic cyanide ligand to broaden the understanding of interactions between linear ligands and heme proteins. Equilibrium and kinetic studies with cyanide are reported for myoglobin and a model system, the heme octapeptide. The equilibrium constants for the azide adducts of myoglobin and octapeptide are also reported. These studies are intended to probe as quantitatively as possible the effects of steric constraints and simple Coulombic interactions on the dynamics of ligand binding in heme proteins.

Experimental Section

Salt-free myoglobin from horse skeletal muscle was purchased from Sigma Chemical Co. Solutions were made in 0.05 M phosphate buffer, pH 7. Potassium ferricyanide was added to ensure complete oxidation of the myoglobin and then removed by desalting on a Sephadex G-25

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