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FLUORESCENCE PROPERTIES OF A BENZO(a)PYRENE 7,8 DIHYDRODIOL 9,10-OXIDE-DNA ADDUCT. CONFORMATION AND EFFECTS OF INTER-MOLECULAR DNA INTERACTIONS

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Summary

The pyrene-like fluorescence of the covalent benzo(a)pyrene diol-epoxide-DNA complex prepared by reacting 7,8,-dihydrodiol 9,10-epoxy benzo(a)pyrene (BPDE) with DNA in aqueous solution in vitro, has been investigated. It is shown that this fluorescence is sensitive to molecular oxygen, to the concentration of native DNA and to the ionic strength (KCl concentration), but is insensitive to the concentration of denatured DNA. These effects are related to the conformation of the pyrene-like chromophore of BPDE. Most of the fluorescence of a dilute solution of the DNA-bound benzo(a)pyrene derivative originates from binding sites in which the pyrene moiety is not intercalated between the DNA base pairs, but is located on the outside of the DNA double helix.

INTRODUCTION

The utilization of fluorescence spectroscopy to characterize the properties of polycylic aromatic carcinogen-DNA complexes is becoming increasingly useful (1-9). To date, the most widely studied polycyclic aromatic carcinogen has been benzo(a)pyrene (BP). It is well known that in vivo BP is metabolized by the P-450 microsomal enzyme system to a variety of oxygenated derivatives (10,11). One of these metabolic intermediates, 7,8-diol-9,10-oxide benzo(a)pyrene (BPDE), is the major intermediate which binds covalently to nucleic acids in vivo. Fluorescence spectroscopy was among the tools which was utilized to establish that the principal metabolic intermediate of BP which reacted with DNA (or RNA) in living cells and tissues involved the loss of aromaticity at the 7,8,9,10 ring; thus the aromatic moiety of this carcinogen covalently attached to DNA, has a pyrene-like chromophore (1,2). A detailed analysis of the fluorescence properties of such covalent polycyclic aromatic molecule-DNA complexes can provide additional important information about the structure of these complexes (3).

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In this communication it is shown that the pyrene-like fluorescence of these covalent BPDE-DNA adducts in 5mM cacodylate buffer solution at 25°C is strongly dependent on the concentration of native DNA, but is independent of the concentration of denatured DNA. These results indicate that intermolecular DNA-DNA aggregation is occurring which gives rise to a quenching of the fluorescence as the DNA concentration is increased; this intermolecular quenching effect occurs only when the DNA is in its native form, since the addition of denatured DNA has no effect on the fluorescence yield. Consistent with this agreggation model, it is shown that the fluorescence yield at relatively high DNA concentrations can be markedly increased by the addition of KCl; this effect is attributed to decreased intermolecular DNA agreggation effects as the ionic strength of the solution is increased.

The basic observations are consistent with those of Yang et al (5), who showed that the fluorescence yield of covalent BPDE-DNA complexes is increased upon denaturation. However, these workers concluded that such effects are due to an intercalation of a significant fraction of the pyrene chromophores between base pairs of native DNA. In contrast, our results indicate that the pyrene chromophores are located external to the native DNA helix in dilute DNA solution. The results of Yang et al (5) can be explained in terms of intermolecular rather than intramolecular fluorescence quenching by DNA, since the enhancement of the fluorescence upon denaturation of the BPDE-DNA complex, is diminished as the DNA concentration is decreased. It is possible that the pyrene chromophore attached to one DNA molecule, intercalates between base pairs of another DNA molecule, when DNA-DNA agreggation is occuring at high DNA concentrations. Since the intermolecular quenching effect is observed only with native DNA, but not with denatured DNA, this hypothesis is plausible.

The conclusions concerning the conformation of the BPDE-DNA adduct, in which the pyrene moiety is located externally to the DNA helix, is supported by (I) the marked sensitivity of the fluorescence to dissolved molecular oxygen, (II) the sensitivity of the fluorescence to different ions (3), and by (III) electric linear dichroism measurements which show that the in-plane long axis of the pyrene chromophore is oriented at an angle of 35° or less with respect to the axis of the DNA helix (12).

EXPERIMENTAL

The in vitro chemical reaction of DNA and BPDE was similar to the one described by Pulkrabek et al (13). The BPDE was a kind gift of Dr. R.G. Harvey (University of Chicago). Calf Thymus DNA (Worthington Chemicals, hyperchromicity: 40%) was dissolved in 5mM sodium cacodylate buffer (pH-7.1). An ethanol solution (5% final ethanol concentration) of BPDE was added to the DNA solution and incubated for 15 minutes at 37°C. This solution was repeatedly extracted with ether to remove all unreacted and physically bound tetraols formed by the hydrolysis of BPDE.

Under these reaction conditions, essentially one stereospecific adduct is detectable (3), consisting of addition of BPDE at the 10 position to the N-2 position of guanine (however, see also the work of Osborne et al (14), which shows that a labile adduct at the N-7 position of guanine is also formed). Steady-state fluorescence measurements were made either on a Hitachi-Perkin-Elmer MPF-2A or an MPF-44A fluorimeter. Fluorescence decay time measurements were made with a single photon counting system consisting mainly of Ortec components, and which has been described elsewhere (3,4). Relative fluorescence yields as a function of DNA (or KCl) concentration were determined by sequential addition of a concentrated native or denatured DNA solution (or solid KCl) to a 1 x 1 cm quartz cuvette. The fluorescence intensity of the BPDE-DNA complex in this cuvette was compared to another cuvette containing exactly the same concentration of the BPDE-DNA complex to which no additions of DNA (or KCl) were made.

RESULTS AND DISCUSSION

The lowest electronic singlet-singlet transition (^1L_b) of pyrene is a relatively forbidden one (15), and thus the fluorescence lifetime of pyrene may be several hundreds of nanoseconds long, depending on the physical environment of the molecule. The fluorescence emission of the BPDE-DNA complex exhibits a typical pyrene-like spectrum (Fig. 1), and the decay profile exhibits, in general,

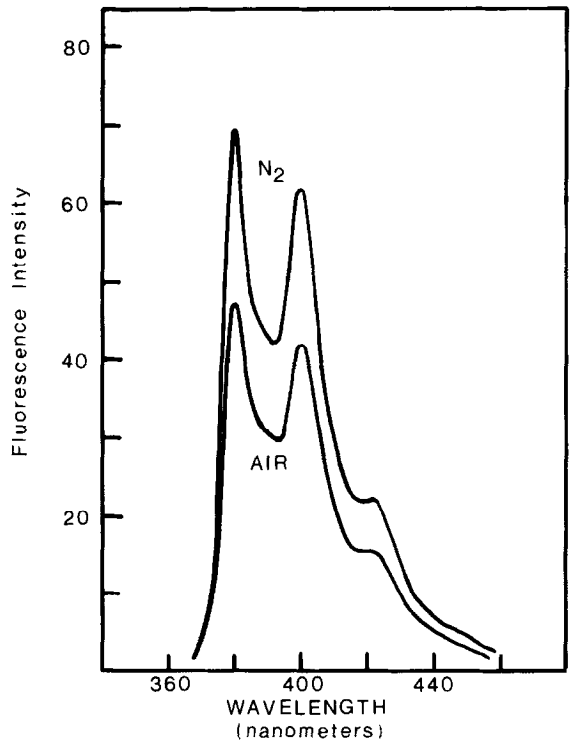

Fig. 1. Fluorescence spectra of a BPDE-DNA complex (0.3% modification, 0.15 A_{260}/ml, 5mM sodium cacodylate buffer, 25°C) in the presence of air (lower curve) and the same sample deaerated by nitrogen bubbling (upper curve).

two components (3). The slowly decaying component is exponential and the calculated lifetime is 200 ns in oxygen-free solution, and 130 ns in air-saturated solution. The same long decay times are exhibited by the tetraol (obtained by hydrolysis of BPDE) in aqueous solution. This tetraol is chemically the same as the covalently bound BPDE molecule, except for the guanosyl moiety attached at the 10-position in the covalent DNA adduct (16). Thus the long fluorescence component of the BPDE chromophore appears to be completely unquenched, even though it is chemically attached to DNA. This may be considered somewhat unusual in the light of the fact that the fluorescence of benzo(a)pyrene which is physically complexed with DNA is strongly quenched, and the fluorescence decay is highly non-exponential; this decay profile can be resolved into two components both of which are characterized by decay times considerably shorter than that of BP itself not bound to DNA (4).

In the case of the BPDE-DNA complex, information can be obtained about the physical location of the aromatic polycyclic molecule, by just observing the effect of oxygen on the fluorescence intensity of the latter. The fluorescence spectra and relative intensities of an air-saturated and an oxygen-free solution are shown in Fig. 1. In oxygen-free solution the fluorescence is enhanced by 47%. Such a large enhancement is not compatible with an intercalation-type conformation, in which the pyrene-like chromophore is sandwiched between adjacent base pairs of DNA. This conclusion may be rationalized on the following basis.

The fluorescence yield as a function of the concentration of a quencher, whose concentration is Q, is described by the Stern-Volmer equation

$$\frac{F_o}{F} = 1 + k\tau_o Q \qquad (1)$$

where F_o and F are the fluorescence yields in the absence and presence of the quencher respectively, τ_o is the lifetime of the fluorescence in the absence of the quencher, and k is the bimolecular encounter rate constant. In water, at room temperature, k has the value of 10^{10} lit $mole^{-1}$ sec^{-1} (17, 18). For ethidium bromide intercalated in DNA in solution at room temperature, Lakowicz and Weber (17) have shown that k is reduced by a factor of 20 as compared to its value in water. Thus, for such an aromatic molecule as ethidium bromide intercalated in DNA, $k\,(DNA) = \frac{1}{20}\, k(H_2O) = 5 \times 10^8$ lit $mole^{-1}$ sec^{-1}. In order to observe measurable effects of oxygen on the fluorescence of intercalated ethodium bromide, Lakowicz and Weber resorted to a high pressure cell and used pressures of oxygen as high as 100 atm. Geacintov et al (4) have shown that similar information can be obtained at ambient oxygen pressures by utilizing as

a probe the triplet excited states of the aromatic hydrocarbons of benzo(a)pyrene and benzo(e)pyrene intercalated in DNA in aqueous solution at room temperature. They also found that the accessibility of these polycyclic aromatic molecules when bound to DNA by intercalation is reduced by a factor of 10-20. Thus, if BPDE were intercalated, and utilizing $k(DNA) = 5 \times 10^8$ lit m^{-1} s^{-1} and τ_o = 200 ns, equation (1) predicts that F_o/F should be 1.03 (the solubility of oxygen at ambient pressures at 25°C is 2.75×10^{-4}M), i.e. a 3% increase in the fluorescence intensity should be observed upon removing oxygen from an air saturated solution. Thus a simple degassing of the solution, and the concomitant effect on the fluorescence intensity, indicates that the fluorescent BPDE cannot have the same simple intercalation type conformation (12) as the physical complex of BP in DNA. Calculation of k from the data shown in Fig. 1, and utilizing τ_o = 200 ns yields a value of $k = 8.5 \times 10^9$ M^{-1} s^{-1}, or just slightly smaller than the value for the BPDE tetraol in aqueous solution. This series of experiments, i.e. the determination of the relative fluorescence intensity in the absence and in the presence of oxygen, indicates that the pyrene-like chromophore is located externally to the DNA helix.

We have discovered that the fluorescence intensity of the BPDE chromophore bound to DNA is a strong function of the concentration of native DNA. The concentration of the DNA may be varied by either adding unmodified DNA to a dilute solution of the complex, or by simply diluting a concentrated solution of the complex. In both cases identical effects are observed. In Fig. 2 it is shown that the fluorescence yield decreases strongly as the DNA concentration of an initially dilute solution is increased by a factor of up to 80; the fluorescence intensity is decreased by a factor of 5. We conclude that this quenching effect is due to intermolecular DNA-DNA interactions and aggregate formation between segments of different molecules of DNA. The fluorescence yield of the pyrene-like chromophore is very sensitive to the formation of these intermolecular aggregates, which is consistent with a conformation in which the pyrene chromophore is located on the exterior of the helix.

Figure 2 also shows the effect of concentration of denaturated DNA on the fluorescence yield. In contrast to the effect observed when the concentration of native DNA is increased, there is no detectable effect of denatured DNA concentration on the fluorescence yield. Addition of native DNA to a sample already containing denatured DNA at a concentration of 3.5 A_{260} units per ml gives rise to the quenching effect observed when native DNA is present only.

The results shown in Fig. 2 suggest that the fluoresence quantum yield increase upon denaturation of the BPDE-DNA complex observed by Yang et al (5) *should* be a function of DNA concentration. That this is indeed the case, is demonstrated in Table I. It is evident that the fluorescence enhancement of

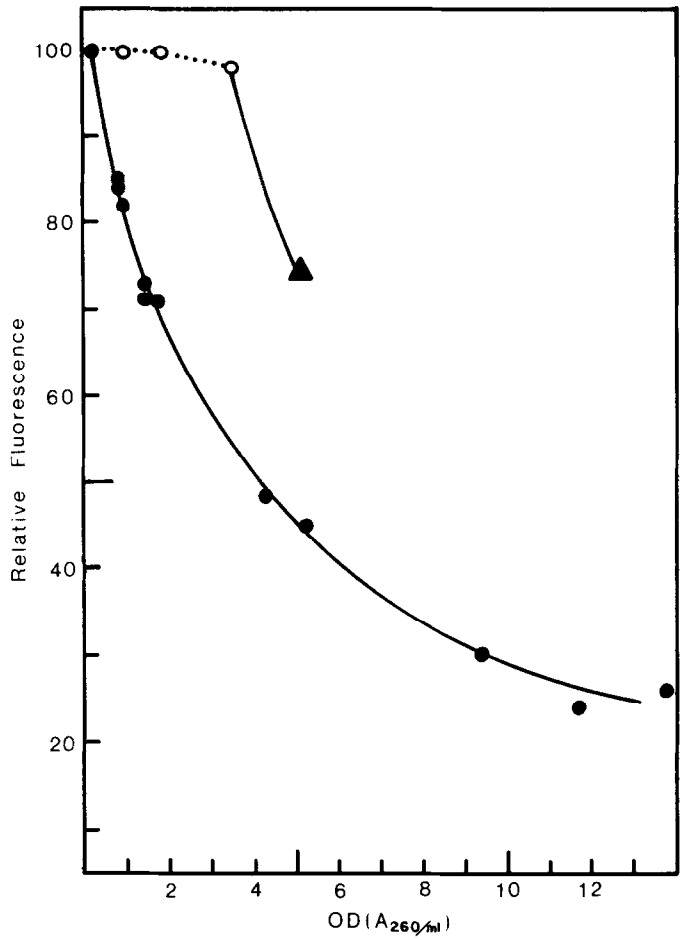

Fig. 2. Relative fluorescence intensity of a 0.3% BPDE-DNA complex (0.17 A_{260}/ml, 5mM sodium cacodylate buffer, 25°C) versus the optical density (absorbance, 1 cm path, 260nm) of added native DNA (●) in the absence of air. Effect of denatured DNA (○) on the fluorescence intensity of a 0.53% BPDE-DNA complex (0.06A_{260}/ml, 5mM sodium cacodylate buffer, 25°C). For a better comparison, the concentration of denatured DNA is based on the absorbance of native DNA. Addition of native DNA to the solution containing denatured DNA at 3.5 A_{260}/ml (▲).

TABLE I

Effect of Denaturation on the Fluorescence Yield of a Covalent BPDE-DNA Adduct (0.58% Modification, 5mM Cacodylate Buffer Solution).

Concentration of DNA (A_{260}/ml)	Relative Fluorescence Yield (Denatured/Native DNA Complex).
0.02	1.02
1.8	1.36
3.7	1.76

the pyrene-like chromophore upon denaturation decreases as the DNA concentration is decreased. These results thus support our interpretation that the fluorescence yield is decreased by intermolecular DNA-DNA aggregation. As these effects are minimized by decreasing the DNA concentration, the fluorescence enhancement factor approaches a few percent only, instead of the value of 150-200% reported by Yang et al. Thus we conclude that in the case of the covalent BPDE-DNA complex, denaturation experiments do not reveal that the pyrene chromophore is intercalated between base pairs, but is a manifestation of intermolecular DNA aggregation and subsequent quenching effects.

These intermolecular DNA interactions are likely to be electrostatic in nature and this should depend on the ionic strength of the solution. This may be conveniently varied by the addition of KCl to the aqueous solution. It is shown in Fig. 3 that the intensity of the fluorescence increases as the KCl concentration is increased to 5 mM buffer to 1.5M KCl by a factor of 3. The effect shown is at $12A_{260/ml}$ where there is a large decrease in yield due to

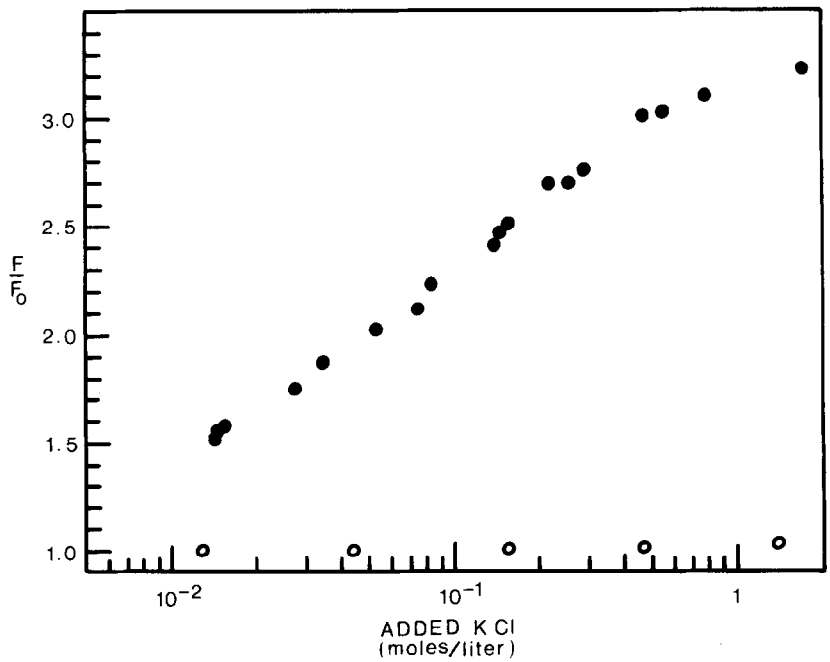

Fig. 3. Ratio of fluorescence intensity in the absence (F_o) and in the presence (F) of added KCl versus concentration of added KCl.

(●) 0.18 A_{260}/ml of 0.3% modified BPDE-DNA (5mM sodium cacodylate buffer, 25°C) with native unmodified DNA added to give a total DNA concentration of 12 A_{260}/ml.

(○) 0.18 A_{260}/ml of 0.3% modified BPDE-DNA - no additional native DNA added.

these intermolecular effects. When the DNA concentration is reduced to a point where the DNA interactions are minimal, there is no KCl effect. We have previously shown (3) that dilute solutions of BPDE-DNA show little or no fluorescence effects when the DNA base binding ions Ag^{+} or Hg^{++} are added to the covalent complexes. It should be pointed out that when these metal ions are added to concentrated DNA solutions, enhancement of the fluorescence results. This is probably due to electrostatic effects as well.

CONCLUSIONS

A detailed analysis of the fluorescence properties of the covalent BPDE-DNA complex can provide valuable information about its conformation. However, a number of factors must be taken into account. The fluorescence yield depends on the concentration of native DNA (at relatively high concentration only), on the ionic strength, and on the concentration of molecular oxygen in solution. These effects are fully consistent with a conformation in which the pyrene-like chromophore of BPDE is located outside of the DNA helix.

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