Vol. 94, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1980 Pages 458-465

FLUORESCENCE STUDY OF (±)-TRANS-7,8-DIHYDROXY-7,8-DIHYDRO-BENZO(A)PYRENE METABOLISM IN VITRO AND BINDING TO DNA

Ola Undeman, Margareta Sahlin, Astrid Gräslund and Anders Ehrenberg

Department of Biophysics, University of Stockholm,
Arrhenius Laboratory, S-106 91 Stockholm, Sweden

Lennart Dock and Bengt Jernström

Department of Forensic Medicine, Karolinska Institutet,
Box 60 400, S-104 01 Stockholm, Sweden

Received April 12,1980

SUMMARY: Fluorescence spectroscopy has been used to characterize the products of (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene bound to DNA after activation by liver microsomes from rats induced by 3-methylcholanthrene. At least two different physico-chemical DNA-carcinogen complexes are formed. One complex is probably formed by a derivative bound on the outside of the DNA helix. Its fluorescence is readily quenched by iodide ions. The fluorescence of the other complex is strongly quenched by DNA. Addition of silver ions increases the total fluorescence of the complexes up to 6 times, indicating reduction of the quenching effect of DNA. These results were found to be in good agreement with those obtained with complexes formed by reaction of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene with DNA.

INTRODUCTION: The metabolism and formation of DNA adducts from the potent carcinogen BP* have been extensively studied. Among the several metabolites that bind to DNA, BPDE has been proposed to be the ultimate carcinogen (1,2). The reaction of BPDE with native DNA appears to be stereoselective in that the (+)enantiomer reacts specifically with the 2-amino group of guanine both in vivo (3-5) and in vitro (6-8).

It has been suggested that the pyrene-like moiety of the BPDE molecule is intercalated between the base pairs in the DNA helix (6,9-11). Observations supporting this hypothesis are, for instance, that specific binding of BPDE to guanine occurs only with the native but not with denatured DNA (6)

*Abbreviations: BP, benzo(a)pyrene; BPDE, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; BP-7,8-diol, (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene; BP-7,8,9,10-tetrol, (±)-7β,8α,9α,10β-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene; HPLC, high performance liquid chromatography.

0006-291X/80/100458-08$01.00/0

and that BPDE causes unwinding of supercoiled SV40 DNA (9). In contrast, other studies using electric linear dichroism (12), fluorescence quenching (13) and optically detected magnetic resonance (14) have provided evidence that the chromophore of bound BPDE is located on the outside of the DNA double helix.

To further elucidate the stereochemistry of the BPDE-DNA complex we have made a fluorescence study of DNA modified by microsomally activated BP-7,8-diol. In parallel experiments we have also studied DNA modified by direct addition of BPDE to an aqueous DNA solution.

MATERIALS AND METHODS:

Incubations. Calf thymus DNA (Sigma type I), BP-7,8-diol (synthesized under NCI contract No. 1-CP-33387 and kindly supplied by IIT Research Institute, Chicago, Ill., USA), microsomes and necessary cofactors were incubated at 37°C for 15 min as previously described (11). One ml of incubation mixture contained the following components: 1 mg microsomal protein, 1 mg DNA, 50 µmoles Tris-HCl (pH 7.5), 250 µmoles sucrose, 10 µmoles Na-EDTA, 1 µmole NADPH and 4 nmoles BP-7,8-diol added in 25 µl acetone. The DNA was isolated as described (11). The extent of modification was approximately one BP-7,8-diol metabolite bound per 1500 DNA bases, as measured using ^{14}C labeled substrate.

The BPDE-DNA adduct was prepared by direct addition of BPDE (same source as for BP-7,8-diol) dissolved in ethanol to an aqueous DNA solution and incubation at 37°C for 15 min. One ml of the reaction mixture contained 1 mg DNA, 500 nmoles BPDE, 0.2 ml ethanol, 15 µmoles NaCl, 1.5 µmoles sodium citrate (pH 7.5). The extent of modification, based on light absorption at 345 nm, was approximately one BPDE residue per 300 DNA bases. DNA samples without the BP-derivatives were always prepared in parallel.

BP-7,8,9,10-tetrol was prepared by HPLC separation of the hydrolysis products of BPDE. All other chemicals were of analytical grade.

Fluorescence measurements. The purified DNA solutions were extracted 10-13 times with 3 volumes of ethylacetate saturated with cacodylate buffer to remove physically bound hydrocarbons. It should be noted that a scrupulous extraction of the DNA samples to remove hydrocarbons that are not stably covalently bound is essential for the results presented in this paper. The DNA was precipitated with ethanol and washed twice with 10 ml of 70% ethanol before drying in vacuum for storage. The hyperchromicity of the DNA denatured with formaldehyde and heat was 40% (15). DNA concentrations were determined by the UV absorption using $\varepsilon_{260nm} = 6600\ M^{-1}\ cm^{-1}$ expressed as moles of nucleotide equivalents per liter per centimeter. Prior to fluorescence measurements the DNA was dissolved in 0.01 M sodium cacodylate (pH 7.0). The DNA solutions were finally diluted and filtered through glass-wool into 1 cm square cells and the fluorescence excitation and emission spectra were recorded on a spectrofluorimeter, described elsewhere (11). Fluorescence quenchers (NaI and $AgNO_3$, Merck) were added in aliquots (50 µl for I^- and 5 µl for Ag^+) to 1.5 ml DNA solution. The solution was bubbled with nitrogen gas saturated with water (O_2 conc. < 10 ppm) for 15 min prior to each measurement. The excitation light causes photochemical reactions of the bound

chromophore, which results in an increasing fluorescence intensity during measurements. The influence of this effect was kept below the detection limit by gently stirring the samples with nitrogen bubbling during the fluorescence measurements. In the quenching experiments the fluorescence intensities for excitation at 332 and 348 nm were measured with the emission monochromator set at 421 nm. At each quencher concentration a blank experiment was carried out with quencher added to unmodified DNA and the intensities thus recorded were subtracted from the sample fluorescence intensities.

RESULTS AND DISCUSSION:

Fluorescence spectra. Fig. 1 shows the fluorescence excitation and emission spectra of BP-7,8-diol metabolites bound to DNA. The corresponding spectra (broken line) of BP-7,8,9,10-tetrol dissolved in water are also shown. The fluorescence data are summarized in Table I, together with the data from synthetically prepared BPDE-DNA.

Microsomal metabolites of BP-7,8-diol bound to DNA and BPDE bound to DNA show almost identical red-shifted pyrene-like excitation and emission spectra. The binding of the pyrene-like chromophore to DNA causes an apparent line broadening and a slight red-shift in the spectra as compared to the

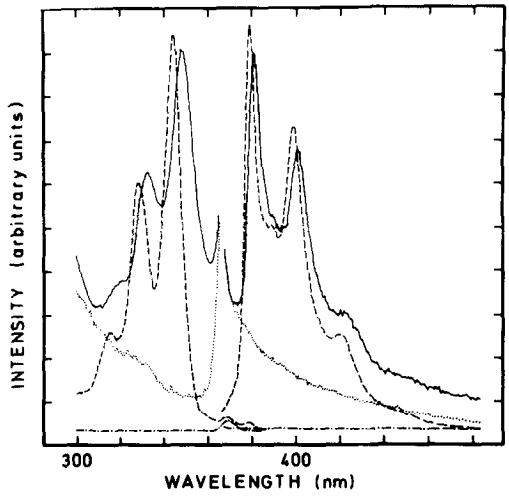

Fig. 1. Fluorescence excitation and emission spectra. (———) Metabolites of BP-7,8-diol bound to DNA, OD_{260nm} = 0.5; (·····) DNA background, OD_{260nm} = 0.5; (-----) 0.1 μM BP-7,8,9,10-tetrol; (-·-·-) solvent background. Samples were prepared in 10 mM sodium cacodylate buffer, pH 7.0, deoxygenated. Excitation spectra were recorded with the emission monochromator set at 421 nm and the emission spectra with excitation at 320 nm. The spectral bandwidth was set at 4 nm.

TABLE I: Fluorescence spectral data. Data were taken from spectra recorded as described in legend to Fig. 1.

	excitation (nm)	emission (nm)
Metabolite of BP-7,8-diol bound to DNA	321, 333, 348	381, 401, 422
Synthetic BPDE-DNA	319, 331, 346	381, 401, 422
BP-7,8,9,10-tetrol	301, 316, 329, 344*	380, 400, 420

*The weak transitions above 344 nm are omitted.

spectra from BP-7,8,9,10-tetrol (Fig. 1). The spectral broadening is indicative of a heterogeneity of DNA binding sites with respect to the microenvironments of the chromophores. This has also been observed by Geacintov et al. in the absorption spectra of BPDE-DNA (12).

The fluorescence lifetime of BP-7,8,9,10-tetrol dissolved in water is relatively long (i.e. 200 ns) and the quantum yield is high (13). In the case of DNA complexes the possibility that chromophores may occur in quenched states has to be considered (16-18). DNA itself quenches the BPDE-DNA fluorescence in a concentration-dependent manner. This has been attributed to DNA-DNA interactions (13,19). To avoid interference from intermolecular DNA quenching we have used dilute DNA solutions. Our measurements show that concentration-dependent effects of DNA on the fluorescence intensity are below the limits of detection when the DNA concentration is < 0.33 OD_{260nm}, corresponding to 50 μM DNA phosphate. Likewise, varying the ionic strength of the solutions (by adding solid NaCl to give final concentrations of 0.01 - 1 M or $MgCl_2$ to give final concentrations up to 0.1 M) had no effect on the fluorescence of DNA at concentrations < 0.33 OD_{260nm}.

Quenching by iodide. Other studies have shown that quenching of the fluorescence from molecules intercalated in DNA by iodide is reduced (20,21), compared to quenching of the free chromophore in solution. This effect was attributed to steric protection of the intercalated molecule from collision with the I^- ion (20). In addition the DNA, which under our experimental conditions is negatively charged (22), should repel iodide ions electrostatically.

Fig. 2 shows the results (in the form of Stern-Volmer plots (23)) of I^- quenching experiments on BP-7,8-diol metabolites bound to DNA, on BPDE-DNA and on BP-7,8,9,10-tetrol dissolved in water. Chloride was added together with the iodide to maintain a constant ionic strength of 0.1 M. Fig. 2 shows that a straight line is obtained only with the BP-7,8,9,10-tetrol dissolved in water. The Stern-Volmer relation is obeyed over the whole iodide concentration range for this sample. For the two other samples, the deviation from a straight line at high I^- concentrations indicates that the DNA-bound chromophore exhibits more than one fluorescence emitting site. This is also evidenced by a 2 nm red shift in the excitation spectrum at the higher I^- concentrations (not shown).

A more dilute BPDE-DNA solution (0.02 OD_{260nm}) gave essentially the same results, indicating that no intermolecular DNA quenching is involved.

Titrations with Ag^+. At a Ag^+:DNA phosphate ratio (r) < 0.2, Ag^+ binds preferentially to the bases of DNA (24). Fluorescence from intercalated molecules

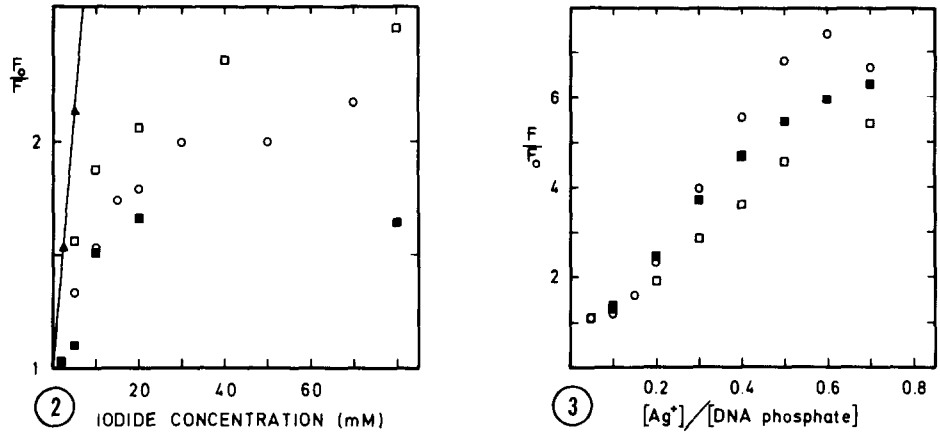

Fig. 2. Stern-Volmer plot of iodide quenching of the fluorescence of BP-7,8-diol metabolites bound to DNA, OD_{260nm} = 0.33 (○); BPDE-DNA, OD_{260nm} = 0.33 (□); BPDE-DNA, OD_{260nm} = 0.02 (■); BP-7,8,9,10-tetrol in water solution (△). The samples were prepared and the quenching measured as described in Materials and Methods. F_o/F is the ratio between fluorescence intensities without and with added iodide ions.

Fig. 3. The effect of Ag^+ ions on the fluorescence of BP-7,8-dihydrodiol metabolites bound to DNA, OD_{260nm} = 0.33 (○); BPDE-DNA, OD_{260nm} = 0.33 (□); BPDE-DNA, OD_{260nm} = 0.033 (■). The samples were prepared and the fluorescence measured as described in Materials and Methods. F/F_o is the ratio between fluorescence intensities with and without added silver ions.

(e.g., BP physically bound to DNA (21)) is strongly quenched by bound silver ions close to the intercalated chromophore.

The results of the titrations with Ag^+ are presented in Fig. 3. Initially, when $r < 0.2$, there is a slight increase in the fluorescence intensity as Ag^+ is added. For $0.2 < r < 0.5$ there is a more pronounced increase. The total net increase in the fluorescence intensity is about 6-fold, both for the DNA complex obtained in the presence of microsomes and for the BPDE-DNA. Titration of dilute BPDE-DNA samples with a DNA concentration of 0.033 OD_{260nm} with Ag^+ gave the same result (within experimental error) as titrations on a BPDE-DNA sample with 0.33 OD_{260nm}. The results may be interpreted in terms of DNA structural changes due to Ag^+ ions at concentrations corresponding to $0.2 < r < 0.5$ (24), driving chromophores from highly quenched states to less quenched ones. The chromophore is not extractable with ethylacetate after addition of Ag^+, revealing covalent binding to DNA.

CONCLUSIONS: Our results show that the fluorescence properties of metabolites of BP-7,8-diol bound to DNA and synthetic BPDE-DNA complexes are qualitatively the same. In both cases we observe the existence of at least two fluorescent states, representing populations of chromophores with different microenvironments. Preliminary fluorescence lifetime measurements resolved into two decay times (25) have given the values 9 ns for a highly quenched state and 80 ns for a less quenched one, similar to results reported by Prusik *et al.* (13). The effects of titrations with I^- and Ag^+ indicate that 70-90% of the chromophores (estimated using the above lifetimes) are in the highly quenched state.

The DNA-quenched states are probably not caused by intermolecular DNA interactions or intramolecular interactions with a part of the DNA helix distant from the binding site, since decreasing the DNA concentration or increasing the ionic strength does not affect the fluorescence. The present results are consistent with strong DNA base-chromophore interactions which

may be at least partially of intercalative nature. Although the strongly DNA-quenched chromophores constitute a large fraction of the total amount, their contribution to the fluorescence is only about 30% (assuming their fraction to be 80% of the total, with lifetimes as stated above). The present results with I^- quenching show that the less DNA-quenched chromophores are exposed to the aqueous phase, i.e. probably bound on the outside of the DNA helix. However, the results do not conclusively show whether and to what extent the strongly DNA-quenched chromophores are accessible to I^- ions. Fluorescence lifetime studies are in progress (25) to further elucidate the stereochemical properties of the bound chromophores.

ACKNOWLEDGEMENTS: We wish to thank Prof. Sten Orrenius and Dr. Joe de Pierre for their helpful advice and support and Mr. Torbjörn Astlind for excellent technical help with the fluorimeter system. This study was supported by the Swedish Cancer Society (Dept. of Biophysics) and by the National Cancer Institute, contract No. 1 RC1 CA26261-01 (Dept. of Forensic Medicine).

REFERENCES

1. Levin, W., Wood, A.W., Yagi, H., Jerina, D.M. and Conney, A.H. (1976) Proc. Natl. Acad. Sci. USA 73, 3867-3871
2. King, H.W.S., Osborne, M.R., Beland, F.A., Harvey, R.G. and Brookes, P. (1976) Proc. Natl. Acad. Sci. USA 73, 2679-2681
3. Ivanovic, V., Geacintov, N.E., Yamasaki, H. and Weinstein, I.B. (1978) Biochemistry 17, 1597-1603
4. Jeffrey, A.M., Jennette, K.W., Blobstein, S.H., Weinstein, I.B., Beland, F.A., Harvey, R.G., Kasai, H., Miura, I. and Nakanishi, K. (1976) J. Am. Chem. Soc. 98, 5714-5715
5. Koreeda, M., Moore, P.D., Wislocki, P.G., Levin, W., Conney, A.H., Yagi, H. and Jerina, D.M. (1978) Science 199, 778-781
6. Meehan, T. and Straub, K. (1979) Nature 227, 410-412
7. Straub, K.M., Meehan, T., Burlingame, A.L. and Calvin, M. (1977) Proc. Natl. Acad. Sci. USA 74, 5285-5289
8. Pulkrabek, P., Leffler, S., Grunberger, D. and Weinstein, I.B. (1979) Biochemistry 18, 5128-5134
9. Drinkwater, N.R., Miller, J.A., Miller, E.C. and Yang, N.-C. (1978) Cancer Research 38, 3247-3255
10. Yang, N.-C., Ng, L.-K., Neoh, S.B. and Leonov, D. (1978) Biochim. Biophys. Res. Commun. 82, 929-934
11. Jernström, B., Orrenius, S., Undeman, O., Gräslund, A. and Ehrenberg, A. (1978) Cancer Research 38, 2600-2607
12. Geacintov, N.E., Gagliano, A., Ivanovic, V. and Weinstein, I.B. (1978) Biochemistry 17, 5256-5262
13. Prusik, T., Geacintov, N.E., Tobiasz, C., Ivanovic, V. and Weinstein, I.B. (1979) Photochem. Photobiol. 29, 223-242
14. Lefkowitz, S.M., Brenner, H.C., Astorian, D.G. and Clarke, R.H. (1979) FEBS Lett. 105, 77-80

15. Grossman, L. (1968) Methods Enzymol. 12, 467-480
16. Boyland, E. and Green, B. (1962) Brit. J. Cancer 16, 347-360
17. Boyland, E. and Green, B. (1962) Brit. J. Cancer 16, 507-517
18. Matsuyama, A. and Nagata, C. (1972) Photochem. Photobiol. 15, 319-321
19. Prusik, T. and Geacintov, N.E. (1979) Biochem. Biophys. Res. Commun. 88, 782-790
20. Nastasi, M., Morris, J.M., Rayner, D.M., Seligy, V.L., Szabo, A.G., Williams, D.F., Williams, R.E. and Yip, R.W. (1976) J. Am. Chem. Soc. 98, 3979-3986
21. Geacintov, N.E., Prusik, T. and Khosrofian, J.M. (1976) J. Am. Chem. Soc. 98, 6444-6452
22. Manning, G.S. (1978) Quart. Rev. Biophys. 11, 179-246
23. Parker, C.A. (1968) Photoluminescence of solutions, Elsevier, Amsterdam
24. Bloomfield, V.A., Crothers, D.M. and Tinoco, I., Jr. (1974) Physical chemistry of nucleic acids, Harper and Row
25. Undeman, O., Gräslund, A., Ehrenberg, A., Astlind, T., Dock, L. and Jernström, B., in preparation