Fluorescence Line-Narrowing Spectral Analysis of in Vivo Human Hemoglobin-Benzo[a]pyrene Adducts: Comparison to Synthetic Analogues

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Abstract: In order to gain insight as to the structure(s) of the adduct formed in vivo between human hemoglobin and the *anti*-diol epoxide (*anti*-BPDE) of benzo[*a*]pyrene (BaP), a series of model compounds was synthesized to investigate the effect on the fluorescence line-narrowing (FLN) spectra of heteroatom substitution at C-10 in BaP tetrahydrotetrol analogues. Spectra taken at 4.2 K by vibronic laser excitation at both 356.9 and 363.4 nm revealed marked differences between BaP tetrahydrotetrols and synthetic thioether, amino, and ester adducts of *anti*-BPDE. Use of these same excitation wavelengths on intact human globin samples obtained from individuals environmentally exposed to ambient levels of BaP yielded vibronic FLN spectra that were virtually indistinguishable from those of a synthetic C-10 carboxylic ester derived from *anti*-BPDE.

Introduction

Many xenobiotics are metabolized to electrophilic carcinogens, which exert their biological effect by reaction with cellular nucleophilic macromolecules. Since physiological concentrations of carcinogens are low, their reactions may be described by pseudo-first-order reaction kinetics. Correlation of protein and DNA adduct formation is therefore theoretically possible. Adduct accumulation in the erythrocyte-maintained protein hemoglobin (Hb), which has a biological lifetime in humans of ca. 120 days, can conform to simple theoretical descriptions. These include a linear relationship between parent compound dose and adduct level.[1,2]

Reactions that result in the formation of a covalent bond between electrophilic compounds and proteins are apparently dependent on the size and/or shape of the alkylating agent. Low molecular weight carcinogens, such as styrene oxide,[3] form products via adduction which takes place as if the protein's constituent amino acids were free in solution rather than constrained, polymer-bound nucleophiles. Larger electrophilic species, such as the metabolites of aflatoxin B_1[4] and 4-aminobiphenyl,[5] exhibit reactivities that appear to be determined more by the tertiary structure of the protein than by the relative nucleophilicities of the protein's side chains.

The interaction of Hb with the electrophilic metabolites of two polycyclic aromatic hydrocarbons (PAH) has been investigated. Fluoranthene binds to the β-125 cysteine sulfhydryl of rat Hb through its isomeric 2,3-diol 1,10b-epoxide metabolites.[6] This reactive nucleophilic amino acid residue is peculiar to rat, guinea pig, and some mouse Hbs.[7] Human Hb apparently does not have a sulfhydryl group that is sterically available to react with PAH epoxides, although slightly smaller electrophiles derived from aminobiphenyl and styrene will form adducts at the β-93 cysteine of human Hb.[8,9] Attempts to characterize in vitro human Hb adducts formed from reaction with (±)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-BPDE),[10] the ultimate carcinogenic metabolite of benzo[*a*]pyrene (BaP),[11] as well as in vivo mouse Hb adducts formed after dosing with BaP,[10] via purification of acidic and enzymatic digests of the protein have been unsuccessful, leading only to isolation and identification of the isomeric BaP 7,8,9,10-tetrahydrotetrols. Our previous work using ^{18}O incorporation has shown that at least 60% of the in vitro human Hb adducts can be attributed to carboxylic esters, which released BaP tetrahydrotetrols upon disruption of the tertiary structure of the protein.[12] In other types of experiments 15% has been accounted for as several proteolytically stable yet structurally unidentified (other than diode array detected UV spectra) C-10 nitrogen adducts.[10] The structure of the adduct(s) formed in vivo with human Hb could only be inferred to be a carboxylic ester from BaP tetrahydrotetrol isomer ratios obtained by varying the pH of proteolysis solutions of globin obtained from individuals environmentally exposed to BaP.[12]

In a previous study we have shown that the UV spectra of synthetic C-10 amino, thioether, and carboxylic ester adducts of *anti*-BPDE exhibited different red shifts in comparison to BaP tetrahydrotetrols, some depending on the solvent in which the spectra were obtained.[10] In order to gain insight as to the structure of the adduct(s) formed in vivo between human Hb and *anti*-BPDE, the synthetic model compounds were analyzed by a method that would more fully explore the electronic differences of these compounds. The sensitive technique of fluorescence line-narrowing (FLN) spectroscopy was chosen to ascertain the effect of heteroatom substitution at C-10 in BaP tetrahydrotetrol analogues. In this study we compare the FLN spectra of these synthetic adducts with those obtained from samples of *intact human globin* to show that not only can *anti*-BPDE–human Hb adducts formed

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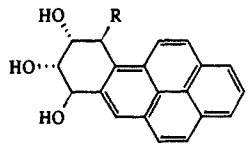

R	
-OH	BP tetrahydrotetrols
$-NHCH_2CH_2NH_2$	EDA-BaP
$-SCH_2CH_2NH_2$	CYS-BaP
$-SCH_2CHCONHCH_2CO_2H$ $NHCOCH_2CH_2CHCO_2H$ NH_2	GSH-BaP
$-OCOCHNHCO_2C(CH_3)_3$ CH_3	ALA-BaP

Figure 1. Synthetic model adducts of benzo[*a*]pyrene *anti*-diol epoxide.

in vivo after environmental exposure to BaP be measured by FLN, but also that the nature of the heteroatom attached at C-10 can be elucidated by this method.

Experimental Section

Instrumentation. The fluorescence line-narrowing spectroscopy system employed in these studies has been described in detail.[13,14] The excitation source was a Lambda Physik FL-2002 dye laser pumped by a Lambda Physik EMG 102 MSC excimer laser. Fluorescence was detected with a Princeton Instruments IRY-1024/G/R/B intensified blue-enhanced gateable photodiode array. Gated detection was accomplished using a Lambda Physik EMG-97 zero-drift controller to trigger a FG-100 high-voltage gate pulse generator, which provides adjustable delay and width of the detector's temporal observation window. Samples were dissolved in 30 μL of 5:4:1 glycerol-H_2O-EtOH in quartz tubes, taken through several freeze–pump–thaw degassing cycles, sealed, cooled to 4.2 K, and probed with the laser in the (0, 1) excitation region. The delay time for fluorescence measurements was 25 ns and the width of the observation window was 60 ns.

Chemicals. Equimolar 7β,8α-dihydroxy-9α,10α-epoxy- and 7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(±)-*anti*-BPDE] was purchased from the NCI Chemical Carcinogen Repository maintained by the Midwest Research Institute (Kansas City, MO). *r*-7,*t*-8,*t*-9,*c*-10-Tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene and *r*-7,*t*-8,*t*-9,*t*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BaP tetrahydrotetrols), *N*-(2-aminoethyl)-*N*-(7,8,9-trihydroxy-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrobenzo[*a*]pyren-10-yl)amine (EDA-BaP), *S*-(7,8,9-trihydroxy-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrobenzo[*a*]pyren-10-yl)-2-thioethylamine (CYS-BaP), *S*-(7,8,9-trihydroxy-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrobenzo[*a*]pyren-10-yl)glutathione thioether (GSH-BaP), and 7,8,9-trihydroxy-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrobenzo[*a*]pyren-10-yl-*N*-*t*-BOC-L-alanate (ALA-BaP) were prepared as previously described.[10]

Human blood samples were obtained from anonymous nonsmoking donors. Red blood cells from 1 mL of whole blood were isolated by centrifugation, washed three times with phosphate-buffered saline, and lysed at 4 °C in 1 volume of double distilled H_2O. Cell debris was removed by centrifugation at 10000*g* for 10 min and the lysate was filtered through a 3 × 30 cm Sephadex G-25 column (H_2O). The eluate was cooled to 4 °C and added dropwise to 300 mL of rapidly stirred 0.015% HCl-Me_2CO maintained at ≤−10 °C. The precipitated globin was pelleted by centrifugation, dried overnight at 25 °C, and stored at −70 °C until analyzed.

Results

Synthetic Model Compounds. The structures of the synthetic model compounds analyzed in this study are shown in Figure 1. The C-10 amino (EDA-BaP), ester (ALA-BaP), and sulfur (GSH- and CYS-BaP) adducts of *anti*-BPDE were originally synthesized to help explain the small red shifts noted in UV spectra of the minor adducts isolated from in vitro reactions of *anti*-BPDE and human erythrocytes.[10] Ester and amino adduct S_2 absorption

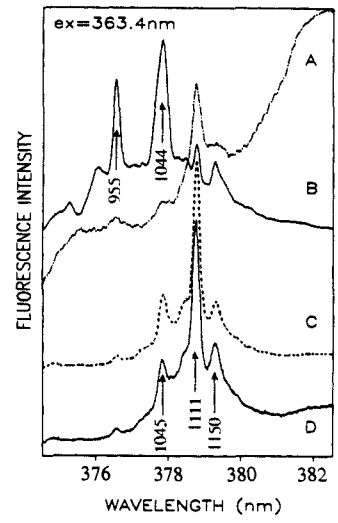

Figure 2. Vibrationally excited fluorescence line-narrowing spectra, obtained by laser excitation at λ_{ex} = 363.4 nm (vibronic excitation), of the synthetic adducts of *anti*-BPDE. ALA-BaP (A; broken line). EDA-BaP (B; upper solid line). (GSH-BaP (C; dashed line). CYS-BaP (D; lower solid line). These and subsequent spectra are labeled in wavenumbers from the laser line.

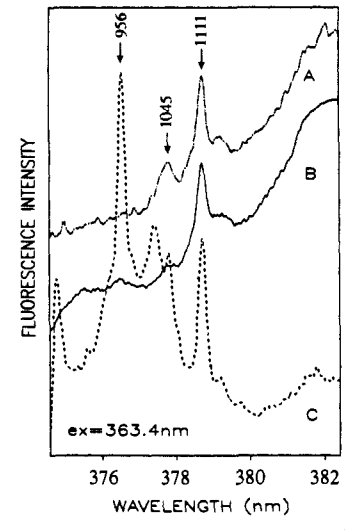

Figure 3. Comparison of the FLN spectra of intact human globin (A) and ALA-BaP (B), the synthetic C-10 ester of *anti*-BPDE, obtained by laser excitation at 363.4 nm, T = 4.2 K. Spectrum C is the spectrum of a 10^{-5} M BaP tetrahydrotetrol–globin mixture obtained by the same excitation.

bands are red shifted 2 nm at room temperature from that of the BaP tetrahydrotetrols, while this band is red shifted 5 nm in the case of thioethers. Since previous work has shown that carboxylic esters of BaP tetrahydrotetrols are readily hydrolyzed in aqueous media,[12] precautions were taken to minimize the contribution of the BaP tetrahydrotetrols to the FLN spectra of the standards.

Figure 2 presents a specific region of the standard FLN spectra obtained with laser excitation at 363.4 nm (vibronic excitation) of the synthetic adducts. The line-narrowed bands are labeled in wavenumbers corresponding to excited-state vibration frequencies of the adducts. The S_1-state energy of BaP tetrahydrotetrols is at higher energy than the C-10 adducts. Thus, the FLN spectrum (Figure 3C) of BaP tetrahydrotetrols noncovalently associated with globin is easily distinguished from the spectra of the synthetic adducts. The S_1 states of the amino and ester adducts are red shifted at 4.2 K ca. 200 and 300 cm^{-1}, respectively, relative to S_1 of the tetrols. Use of the same excitation wavelength, as well as with other vibronic excitation wavelengths (data not shown), on the C-10 sulfur adducts (Figure 2C and D) revealed that the S_1 state of GSH-BaP is at slightly lower energy than that of CYS-BaP. The S_1 energy levels of the sulfur adducts

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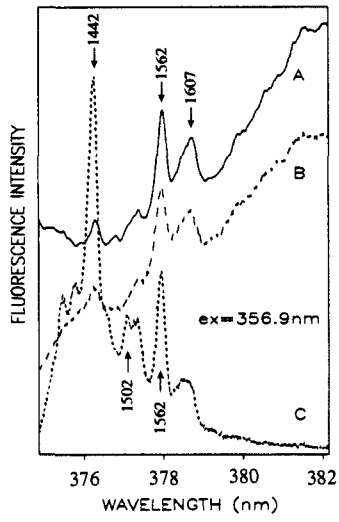

Figure 4. Comparison of the vibrationally excited FLN spectra (λ_{ex} = 356.9 nm, T = 4.2 K) of intact human globin (A), ALA-BaP (B), and the BaP tetrahydrotetrol–globin mixture (C).

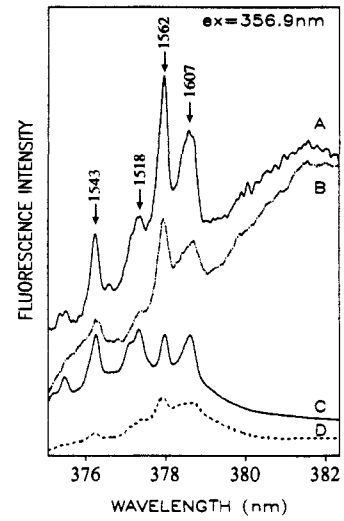

Figure 5. FLN spectra (λ_{ex} = 356.9 nm, T = 4.2 K) of the intact human globin sample (A), ALA-BaP (B), and EDA-BaP (C) after storage at −10 °C for several weeks. Spectrum D is generated by subtracting the contributions of B and C from A and smoothing.

are intermediate to those of the ester and amino adducts. It should be pointed out that these distinctions would be impossible by conventional spectroscopic means. These differences in energy levels mean that the FLN spectra of BaP ester, amino, and thioether adducts can be resolved from BaP tetrahydrotetrol spectra, as well as from themselves, even in mixtures.

The general red shift of the synthetic adducts relative to the tetrols is similar to the 100–300-cm^{-1} red shift observed in the FLN spectra of DNA adducts formed from reaction with both *anti*- and *syn*-BPDE.[14,15]

When adducts of BPDE are no longer covalently attached to globin and exist as BaP tetrahydrotetrols mixed with the protein, the (0, 0) origin of fluorescence is blue shifted and a distinction between covalently (adducts) and noncovalently (tetrols) bound pyrene chromophores is straightforward by FLN spectroscopy. We hypothesized that, since spectra of the three different types of C-10 adducts (S, N, and O) of *anti*-BPDE have clear qualitative differences, these standard spectra should be of use in the analysis of in vivo adducted human Hb.

FLN Spectral Analysis of in Vivo Human Hb–BaP Adducts. It has been demonstrated that, with the present level of detection (ca. 1 adducted base per 10^8 base pairs in ca. 100 μg of DNA), FLN spectroscopy can be used for analysis of in vivo exposed samples.[13,15] For example, FLN analysis of DNA extracted from fish dosed with BaP showed that, at high doses, the major adduct formed was from the syn isomer of BPDE, while at lower doses the ratio of anti to syn products was higher.[15,17] Thus FLN is also capable of assisting in the assignment of the stereochemistries of adducts.

The FLN spectra of the synthetic adducts (vide supra) have been used to aid in the identification of the major human Hb adduct of BPDE. As stated in the introduction, only the BaP tetrahydrotetrols from *anti*-BPDE-derived adducts have been isolated from in vivo exposed human Hb. These tetrols apparently arise from ester adducts in the protein. We obtained 1-mL blood samples from nonsmoking donors who were exposed only to ambient levels of environmental BaP. Hb was purified by gel filtration and the globin was precipitated. The majority of the protein was processed by a previously described method for BaP adduct analysis.[18] A representative sample found to contain 8 pmol of pyrene-like fluorescence per gram of protein was used for further characterization by FLN spectroscopy. Figure 3 is the comparison of the FLN spectra of *3.5 mg of the intact globin sample* (i.e., a 30-fmol sample of pyrene-like compounds; this is particularly noteworthy since 1 mL of whole human blood yields 130–200 mg of globin) and the synthetic ester ALA-BaP, both obtained by 363.4-nm laser excitation. The spectra are virtually indistinguishable and it is apparent that the major adduct is an *anti*-BPDE-derived ester of human Hb.

Figure 4 depicts an additional comparison of FLN of intact human globin (Figure 4A) and ALA-BaP (Figure 4B) obtained by vibronic laser excitation further into the S_1 manifold (excitation at 356.9 nm). These spectra are also virtually indistinguishable. Figure 4C is the FLN spectrum obtained by the same laser excitation of BaP tetrahydrotetrols mixed with globin. This spectrum is clearly different from those of both the synthetic and in vivo formed esters.

Small contributions from C-10 nitrogen adducts cannot be excluded from the FLN spectra we have obtained (note the 1044- and 1045-cm^{-1} lines in Figures 2B and 3A). In samples that were stored at −10 °C for several weeks (in the 5:4:1 glycerol–H_2O–EtOH solvent mixture), an apparent increase in the relative amount of amino-type adducts was observed in the resulting FLN spectra (see Figure 5). It is a temptation to state that this increase in signal is confirming evidence of amino adduct formation from BPDE. However, due to the known mechanism of BPDE-derived ester hydrolysis in neutral media, whereby the PAH residue is released as a C-10 cation,[12] it is a distinct possibility that the amino-like adducts are an artifact of storage in a milieu where reaction of the C-10 cation with H_2O does not occur as readily as does reaction with the protein's abundant amino nucleophiles. This extended storage of the sample in an aqueous, denaturing (EtOH–glycerol) environment apparently promoted the unimolecular decomposition of many of the ester adducts with the subsequent reaction of the C-10 carbocation with other amino acids in the local environment.

Figure 5D is shown in support of the hypothesis that the majority of the non-ester adducts in the sample stored dissolved in the alcohol–H_2O solvent mixture are artifacts. This spectrum is the computer-generated result of subtracting Figure 5B and C from A and is apparently equivalent to the FLN spectrum of the synthetic thioether adducts when excited at 356.9 nm (data not shown). Since there is no evidence supporting the formation of sulfur adducts upon reaction of *anti*-BPDE with human Hb, this type of adduct must originate from carbocations released from esters.

Acrylamide, a known quencher of chromophores situated on the external (H_2O accessible) portions of macromolecules,[16,19] was

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used with the intact globin sample to determine the site of adduction. In agreement with previous speculation,[12] no diminution of the ester signals was noted, suggesting that the adduct is protected in a hydrophobic portion of the Hb. Simple mixing of the BaP tetrahydrotetrols with globin followed by the acrylamide treatment resulted in a complete loss of tetrol fluorescence, suggesting a not so surprising hypothesis that the interaction of BaP tetrahydrotetrols with hemoglobin differs from that of BPDE with the protein.

Discussion

A particularly important aspect of this research has been to determine whether human Hb adducts formed in vivo could be attributed to a specific structural type. Previous mechanism-based isotope incorporation experiments have suggested that carboxylic esters are the major adducts formed by BPDE both in vitro and in vivo.[12] The remarkably straightforward results from the present study confirm the previous findings. Despite the low adduction level present in the globin sample, its FLN spectrum exhibits adequate signal to noise for confident structural assignments. As expected for individuals environmentally exposed to BaP, the major adduct analyzed by our system is derived from BPDE. This is confirmed by comparison of the FLN spectrum of the intact globin adduct to that of a synthetic ester adduct of *anti*-BPDE. The two spectra are essentially the same, demonstrating that the esters are likely candidates for globin adduct formation in vivo. Additional support for these assignments is obtained by noting the similarities that occur at various excitation wavelengths. The spectral evidence clearly is in support of BPDE esterification taking place at one or more carboxylate sites in human hemoglobin.

Experimental data collected on adducted globin demonstrated the utility of acrylamide in quenching pyrene moieties that adopt a conformation external to the protein where water is available. This is similar to DNA where BPDE and the BaP tetrols form external complexes and quasi-intercalated complexes.[19,20] Acrylamide, when dissolved in water and mixed with adducted globin, does not quench the fluorescence from oxygen or nitrogen type adducts formed in vivo with human Hb. On the other hand, free tetrols mixed with the globin samples are quenched, demonstrating that the BaP tetrols are adopting external conformations. Similar fluorescence quenching has been used to distinguish stereoisomers of a given metabolite bound to a particular nucleic acid base.[16] In future studies we hope to use FLN spectroscopy to determine the stereochemistry of the active metabolite of BaP that alkylates hemoglobin in vivo. Finally, we would like to add that after all stable adducts are decomposed and/or hydrolyzed to BaP tetrols the expected FLN spectrum would appear as the one presented in Figures 3C and 4C: the BaP tetrahydrotetrols + globin mixture, spectra that are very different from those of BPDE adducts.

These studies have shown again that FLN spectroscopy is a viable technique for structural identification. It should be a powerful tool for future structural analysis in the characterization of the origin and nature of different human macromolecular adducts. Whether BPDE–DNA adducts are present after in vivo exposure depends on many additional factors, like DNA repair and the rate of cell proliferation.[21] BPDE–protein adducts are not considered to be pathological lesions, but they provide a useful resource in molecular dosimetry of chemical carcinogens. Our spectroscopic studies show that the major globin adduct is an ester, indicating that the adduct formation occurs either at the C termini of the α or β chains or the side chain of aspartate or glutamate in hemoglobin.

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Communications to the Editor

On the Composition of Yamamoto's Reagent: "RCu·BF_3"

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In 1978, a new class of organocopper reagents derived from treatment of CuI with an organolithium (1 equiv) followed by BF_3·Et_2O (1 equiv) was introduced as a means of effectively displacing allylic leaving groups.[2] More recently, these species have found favor as valued Michael donors in couplings with α,β-unsaturated ketones, esters, and even acids.[3] Their composition, originally scribed as $RBF_3^-Cu^+$,[2] is now more commonly written as "RCu·BF_3" (**1**) to reflect the metathesis between an RLi and CuI (eq 1).[3] The role of the byproduct LiI in the subsequent chemistry of **1** has always been ignored. We now report, by virtue of both spectroscopic and chemical experiments, that the success of Yamamoto's reagent is intimately tied to the presence of lithium iodide in the medium and that "RCu·BF_3" is in fact *not* the species that effects C–C bond formation.

$$\text{RLi} + \text{CuI} \longrightarrow \text{RCu} + \text{LiI} \xrightarrow{BF_3} \text{"RCu·}BF_3\text{"} + \text{LiI} \qquad (1)$$

The ^{1}H NMR spectrum of MeCu·LiI + BF_3 (from CuI + MeLi/Et_2O, 0.15 M in THF at −80 °C, then BF_3·Et_2O) shows, in addition to a major broad singlet at δ −1.25, another minor peak at δ 0.15. Upon warming to −40 °C, the peak at δ −1.25 is rapidly replaced by a new signal at δ −0.77. All three signals are observable at −80 °C if Me_2S (3 equiv) is present initially to maximize solution homogeneity (Figure 1).[4] As more LiI is added to the Yamamoto recipe (eq 1), the peak at δ 0.15 grows in still further. Once an additional 2 equiv of LiI have been added to MeCu·LiI + BF_3, the downfield signal is all that remains at −80

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(4) MeCu·LiI itself, 0.15 M in 10% HMPA/THF at −80 °C, gives only the signal at δ −1.25.

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