

Benzo[*a*]pyrene *anti*-Diol Epoxide Covalently Modifies Human Serum Albumin Carboxylate Side Chains and Imidazole Side Chain of Histidine(146)

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Abstract: Human serum albumin was reacted with (\pm)-*r*-7,*t*-8-dihydroxy-*t*-9,*t*-10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-BaPDE) in vitro and extracted to remove byproducts not covalently bound to the protein. Enzymatic digestion of the adducted protein in H₂¹⁸O at pH 8.2 and pH 10.0, followed by analysis of the released 7,8,9,10-tetrahydrotrifols by positive chemical ionization mass spectrometry for ¹⁸O incorporation, revealed that carboxylic esters are formed by the epoxide. Analysis by HPLC/UV of the protein digest indicated that esters are the major product formed. Two additional stable products were also observed, accounting for 22 and 8% of chromatographed material. These were identical in UV absorption spectral characteristics with synthetic *N*^{im}-histidine-*anti*-BaPDE adducts. Amino acid analysis of the peptide portion of the major product, in combination with its FAB mass spectrum, was consistent with a composition of histidine, proline, and tyrosine, while like analysis of the minor adducted peptide was consistent with a composition of histidine and proline. The first combination of amino acids occurs only once within the sequence of human albumin as His(146)-Pro(147)-Tyr(148). The second could be a subsequence of the first or correspond to His(338)-Pro(339) or His(440)-Pro(441). When synthetic His-Pro-Tyr was reacted with *anti*-BaPDE, a product which was chromatographically and spectrally (UV, FAB-MS) identical with the material isolated from alkylated albumin was formed in low yield. Reaction with fluorescamine followed by acid-catalyzed rearrangement of the products and analysis of the fluorescence spectra from the resulting materials revealed that the adducts in the protein resulted from alkylation of the imidazole τ -nitrogen of histidine. These results indicate that, in addition to the unknown amino acids esterified, His(146) and possibly His(338) or His(440), the former two of which are in a previously recognized binding site for certain covalent and noncovalent bulky aromatic ligands, are alkylated by *anti*-BaPDE to form enzymatically stable adducts.

Introduction

Benzo[*a*]pyrene (BaP) is an environmentally prevalent procarcinogen, metabolically activated by cytochromes P450 to the carcinogen *r*-7,*t*-8-dihydroxy-*t*-9,*t*-10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-BaPDE),¹ which alkylates nucleophilic sites on macromolecules at its highly electrophilic C-10 position. The sites and types of adducts formed by *anti*-BaPDE on both DNA and human hemoglobin have been characterized. The epoxide primarily alkylates N-2 of guanine in DNA, preferentially at AGG and CGG triplets,² while it has been shown to covalently modify human hemoglobin at the side chain carboxylate of Asp(47)- α .³ The hemoglobin ester adduct is stable as long as the protein retains its tertiary/quaternary structure.⁴ This ester is readily cleaved under denaturing conditions to yield BaP 7,8,9,10-tetrahydrotrifols (*trans*- and *cis*-BaPT), which can be quantified by GC-MS. Hemoglobin adducts of BaPDE have thus been used as a measure of active metabolite reaching the circulatory system in humans.^{5,6}

Since erythrocytes do not possess the metabolic capacity to generate *anti*-BaPDE, the observation of hemoglobin adducts of *anti*-BaPDE in vivo implies transport of the metabolite from a metabolically competent cell through plasma to erythrocytes. Serum albumin is thus almost certainly also exposed to *anti*-BaPDE, and adducts with serum albumin should prove to be useful as biological dosimeters for BaP exposure.⁷ Previously reported studies on the interactions of *anti*-BaPDE with human serum albumin have not addressed the question of the type(s) or structure(s) of adducts formed by this epoxide with the protein, although adduction is apparently more complex than that seen with hemoglobin.^{8,9} A recent report has communicated an attempt at the use of serum albumin as a dosimeter for BaP exposure,¹⁰ although no attempt was made to determine the structure(s) of the adduct(s), nor if the adducts measured were indeed generated

from BaP or from metabolic products of other polycyclic aromatic hydrocarbons. The possibility of the use of serum albumin-BaPDE adducts in molecular dosimetry studies prompted us to perform further detailed investigations to define the structures.

In this paper we present evidence from isotope incorporation experiments and mass spectrometric analyses for two types of adducts formed by *anti*-BaPDE with serum albumin obtained by analysis of products generated by degradation of the alkylated protein. In a related paper¹¹ we describe vibronic spectroscopic analysis of the partially degraded and undegraded, alkylated protein to verify these structures.

Experimental Section

Instrumentation. High-performance liquid chromatography (HPLC) was performed on a Hewlett-Packard 1090 equipped with a Hewlett-Packard 1040 diode array UV detector. A gradient of 0.1% CF₃CO₂H/H₂O (solvent A)-0.1% CF₃CO₂H/CH₃CN (solvent B) (time = 0, 85% A; 10 min, 75% A; 34 min, 72% A; 35 min, 72% A; 65 min, 70% A; 75 min, 100% B), at flow rates of 1.5 or 4.2 mL/min, was used with either a Waters 3.9 mm \times 30 cm or 7.8 mm \times 30 cm μ Bondapak C18 column, respectively.

Gas chromatography-mass spectrometry (GC-MS) was performed on a Hewlett-Packard 5987A mass spectrometer. Chemical ionization experiments were performed with CH₄ as the reagent or moderating bath

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gas. This instrument's operation has previously been detailed.⁵

Fast atom bombardment mass spectrometry (FAB-MS) of peptides¹² was carried out in the first (MS-1) of two mass spectrometers of a tandem mass spectrometer (JEOL HX110/HX110) at an accelerating voltage of 10 kV and a resolution of 1:2600. Single scans were acquired at a rate to scan m/z 0–6000 in 1.0 min, and with 100–300 Hz filtering. For calibration (CsI), Cs^+ cluster ions were used. The JEOL Cs^+ gun was operated at 26 kV. Tandem mass spectrometry was carried out by using all four sectors in $\text{E}_1\text{B}_1\text{E}_2\text{B}_2$ configuration.¹³ Collision induced dissociation (CID) of protonated peptide molecules, selected with MS-1, took place in the field-free region after B_1 . The collision cell potential was held at 3.0 kV and the ion collision energies were at 7.0 keV. He was the collision gas and was introduced into the collision chamber at a pressure sufficient to reduce the precursor ion signal by 65–70%. The CID mass spectra were recorded with 100 Hz filtering at a rate to scan from m/z 0–6000 in 1.5 min. MS-1 was operated at a resolution adjusted so that only the ^{12}C -species of the protonated adduct molecule to be analyzed was transmitted. MS-2 was operated at a resolution of 1:1000, and was calibrated with a mixture of CsI , NaI , KI , RbI , and LiCl .¹⁴

Room temperature fluorescence measurements were taken on a Spex Fluorolog 2 system consisting of two 0.22 m double monochromators, a Hamamatsu R928 photomultiplier tube held at -30°C by a Products for Research, Inc. TE-177-RF005 thermoelectric crystal, a 450-W xenon lamp as the excitation source, and Spex DM3000F software. Sample concentrations of 1–10 nM were examined.

Adduction of Serum Albumin with *anti*-BaPDE. Human serum albumin (fatty acid-free, Sigma, 80 mg, 1.2 μmol) was dissolved in 2 mL of 10 mM phosphate buffered normal saline (PBS, pH 7.4) in a 5-mL silanized vial equipped with a magnetic stir bar. This solution was treated with $[7\text{-}^{14}\text{C}]\text{-anti-BaPDE}$ (NCI Chemical Repository maintained by Chemsyn Science Laboratories, 53 mCi/mmol, 0.153 μmol , 8.11 μCi) in 50 μL of 19:1 THF- Et_3N . The resulting mixture was stirred at 37°C for 2 h. The reaction mixture was extracted with EtOAc ($4 \times 2\text{ mL}$) and H_2O -saturated 1-BuOH ($4 \times 2\text{ mL}$) then lyophilized. Control experiments with albumin spiked with ^{14}C -labeled BaP tetrols verified that this extraction procedure would remove all noncovalently bound residues. The yield of alkylation was 68% based on *anti*-BaPDE as determined by radioactivity (i.e., 8.7% of the protein was alkylated). The resulting solid was analyzed for ester content (vide infra).

A larger scale reaction was performed as above with 0.8 g (12 μmol) of albumin in 20 mL of PBS (pH 7.4) and 0.5 mg (1.5 μmol) of nonradioactive *anti*-BaPDE (NCI Chemical Repository maintained by the Midwest Research Institute). The reaction mixture was extracted with EtOAc ($4 \times 20\text{ mL}$) and H_2O -saturated 1-BuOH ($4 \times 20\text{ mL}$), the pH was adjusted to 8 with 2 N NaOH, and the protein was digested with two 10-mg batches of Pronase E (vide infra). The solution was made 50 mM with HCO_2NH_4 , its pH was adjusted to 8.5 with NH_4OH , and the peptides containing *anti*-BaPDE-derived adducts were concentrated by *cis*-diol affinity chromatography on immobilized phenylboronic acid (Affigel 601, Bio-Rad). The covalently retained fraction was eluted with 1% HCO_2H . Fractions containing the adducts were analyzed for amino acid composition by 24 h hydrolysis in 6 N HCl followed by formation of and HPLC analysis of the amino acid PTH derivatives. Sequences were determined by Edman degradation at the Harvard Microchemistry Facility, Cambridge, MA. Remaining materials were analyzed both by fluorescence line narrowing spectroscopy¹¹ and FAB-MS. The samples for FAB-MS analysis were derivatized in 0.3–0.5 mL of 1.5:1 pyridine- $(\text{CH}_3\text{CO})_2\text{O}$ at room temperature for 2 h and then dried under vacuum.

^{18}O Incorporation Experiments. The 80-mg sample of dried $[7\text{-}^{14}\text{C}]\text{-anti-BaPDE}$ -adducted human serum albumin was dissolved in 99% H_2^{18}O (2 mL, Isotec) and split into two equivalent fractions, each in a silanized vial equipped with a magnetic stir bar. One fraction was made 0.5 M with NH_4HCO_3 (pH 8.2); the other fraction was made 0.5 M with K_2CO_3 and taken to pH 10 with diisopropylethylamine. Pronase E (Sigma, 5 mg) was added to each fraction. The digest solutions were stirred at 37°C for 6 h. The pH of each solution was readjusted to its starting value with the reagents listed above. Another 5 mg of Pronase E was added, and the digestions were continued an additional 12 h at 37°C . The completed digests were each diluted to 20 mL with PBS and then extracted with EtOAc ($4 \times 20\text{ mL}$). Each set of organic layers was pooled, dried (Na_2SO_4), filtered, and evaporated to dryness under a stream of N_2 . The *anti*-BaPDE-derived analytes remaining in the aqueous layers from each pH experiment were concentrated on a

CNBr-activated Sepharose 4B column covalently modified with the 8E11 monoclonal antibody, which binds BaPDE adducts and tetrols,⁵ eluted from the column with 9:1 CH_3OH - H_2O , and dried on a Speed-Vac centrifugal evaporator. The organic and immunoaffinity-recognized extracts were combined, resuspended in the starting HPLC solvent mixture, and analyzed by HPLC. Fractions were collected and lyophilized. Those corresponding in retention time to the *trans*- and *cis*-tetrols and *anti*-BaPDE were treated with *N*-trimethylsilylimidazole in pyridine (Trisyl-Z, Pierce), and their molecular ion clusters were analyzed by GC-MS via selected ion monitoring in the positive chemical ionization mode.

Synthesis of $\text{N}^{\text{im}}\text{-(7,8,9-Trihydroxy-}r\text{-7,}t\text{-8,}t\text{-9,}c\text{-10-tetrahydrobenzo[a]pyren-10-yl)histidines}$. L-Histidine monohydrochloride monohydrate (Eastman Organic, 200 mg, 391 μmol) was dissolved in H_2O (1 mL) in a silanized vial. The pH was adjusted to 7.5 with 2 N NaOH, and *anti*-BaPDE (NCI Chemical Repository maintained by Midwest Research Institute, 10 mg, 33 μmol) in 0.75 mL dry THF was added under N_2 in 50 μL aliquots over 5 min. The mixture was stirred at 37°C 2 h, diluted with 10 mL of H_2O , and washed with EtOAc ($5 \times 20\text{ mL}$). The organic layers were combined and extracted with 0.1% $\text{CF}_3\text{-CO}_2\text{H}$. The aqueous layers were combined, lyophilized, redissolved in 50 mM HCO_2NH_4 (pH 8.5), and passed through a 20-g phenylboronate *cis*-diol affinity column equilibrated in the same solvent. The column was washed with the solvent (100 mL), bound material was eluted with 200 mL of 1% HCO_2H , and the eluate was neutralized with 2 N NH_4OH and lyophilized. Final purification was by C-18 HPLC, which yielded two major products. Determination of τ - or π -nitrogen alkylation was made by acidic rearrangement followed by fluorescence analysis (excitation at 380 nm, emission at 458 nm and excitation at 345 nm, emission at 378 nm) of carefully measured equimolar amounts of each adduct's α -amino fluorescamine product.¹⁵ CID spectrum of underivatized N^{r} -derivative (m/z , % relative to m/z 257) 303 (76), 285 (34), 257 (100) due to losses of histidine, histidine + H_2O , and histidine + H_2O + CO, respectively; CID spectrum of underivatized N^{t} -derivative (glycerol) (m/z , % relative to m/z 257) 303 (69), 257 (100) due to the same losses as with the N^{r} -derivative.

Synthesis of His-Pro-Tyr and Its $\text{N}^{\text{im}}\text{-(7,8,9-Trihydroxy-}r\text{-7,}t\text{-8,}t\text{-9,}c\text{-10-tetrahydrobenzo[a]pyren-10-yl) derivatives}$. The peptide was synthesized on a 0.1 mmol scale with a $\text{N}^{\alpha}\text{-(9-fluorenylmethyloxy)-carboxyltyrosine } O\text{-}t\text{-tert-butyl ether}$ Wang carboxylate resin (Du Pont) and $\text{N}^{\alpha}\text{-Fmoc amino acids}$. Histidine was additionally N^{im} -trityl protected. Protecting Fmoc groups were removed with 1:1 piperidine-DMF. Active esters were formed with benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP, Richlieu) and diisopropylethylamine. The completed peptide was deprotected and cleaved from the resin by treatment with 19:1:1 $\text{CF}_3\text{CO}_2\text{H-H}_2\text{O-HSC-H}_2\text{CH}_2\text{SH}$. The peptide was precipitated in 30:1 $\text{Et}_2\text{O-CF}_3\text{CO}_2\text{H}$, dissolved in H_2O , exhaustively washed with Et_2O and EtOAc , lyophilized, and purified by HPLC. Yield was 84%: FAB-MS (m/z , %) 416.1 (M + H, 100), 438 (M + Na, 30), 831.3 (dimer + H, 5), 853.3 (dimer + Na, 3); CID (glycerol, peracetylated, m/z , % relative to iminoHis) 436.2 (y_3 , 49), 364.2 (a_3 , 12), 249.1 (a_2 , 8), 180.1 ($b_1\text{-AcHis}$, 84), 152.0 (a_1 , 78), 110.0 (iminoHis, 100), 69.9 (iminoPro, 40).

The *anti*-BaPDE imidazole adducts of this peptide were formed from the reaction of the epoxide (0.1 mg, 330 nmol) in 50 μL of 19:1 THF- Et_3N with the peptide (20 mg, 48 μmol) in 1 mL of H_2O , pH 6.5, at 37°C with stirring for 2 h. The yield was ca. 0.1% as determined by HPLC, with the remainder being the tetrol hydrolysis products as well as minor products from reaction at the α -amino nitrogen and carboxylate oxygens, as determined by shifts in the S_2 absorption bands of their UV spectra. Each of the imidazole alkylated products gave the expected fluorescence after acidic rearrangement of their fluorescamine derivatives.

Synthesis of 10-Ethoxy-7,8,9-trihydroxy-*r*-7,}t\text{-8,}t\text{-9,}c\text{- and -}t\text{-10-tetrahydrobenzo[a]pyrenes. *anti*-BaPDE (1 μg , 3.3 nmol) in 10 μL of 19:1 THF-diisopropylethylamine was added to anhydrous EtOH (1 mL, 17 mmol) in a silanized vial and allowed to stand at room temperature for 10 days. The yield was quantitative. The ratio of *trans*-*cis* products was 10:1 based on GC analysis of the triTMS derivatives. UV absorption spectra of these compounds were indistinguishable from those of BaP tetrahydrotetrols (S_2 absorption maximum at 343 nm): *trans* product, EI-MS (triTMS, m/z , %) 564 (M^+ , 4), 404 (44), 360 (18), 331 (17), 191 (100); PCI-MS (CH_4 , triTMS, m/z , %) 593 (M + 29, 4), 595 (M + H, 28), 265 (45), 221 (66), 191 (100); NICI-MS (CH_4 , triTMS, m/z , %) 564 (M^+ , 14), 446 (98), 402 (100); *cis* product, EI-MS (triTMS, m/z , %) 564 (M^+ , 4), 404 (50), 360 (100), 331 (60), 191 (80); PCI-MS (CH_4 , triTMS, m/z , %) 595 (M + H, 28), 265 (64), 221 (18), 191 (100); NICI-MS (CH_4 , triTMS, m/z , %) 446 (100), 402 (53).

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Table I. Product Ratios^a and ¹⁸O Incorporation^b into the 7,8,9,10-Tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene Isomeric Hydrolysis Products of Human Serum Albumin Adducted with *anti*-BaPDE in Vitro

	pH	
	neutral 7.2–8.3 <i>trans</i> / <i>cis</i>	basic 9.8–10 <i>trans</i> / <i>cis</i>
isomer ratio of tetrols	2.9:1	3.8:1
% ¹⁸ O incorporated into tetrols	22:43	0:0

^a Determined by HPLC with UV detection at 343 nm. ^b Determined with methane positive chemical ionization selected ion monitoring mass spectrometry of the molecular ion cluster of the *tetrakis*(trimethylsilyl) derivatives of the tetrols. ^c *Trans* and *cis* refer to the relative stereochemistry of the substituents on C-9 and C-10 of the 7,8,9,10-tetrahydrobenzo[a]pyrene residue.

Results

Ester Adducts. Human serum albumin was alkylated with *anti*-BaPDE in vitro under physiological conditions and then extracted to remove BaP tetrols which might form from unstable adducts as well as those formed by reaction of *anti*-BaPDE with water. Complete digestion of the protein was performed with Pronase E in >95% isotopic purity H₂¹⁸O at ca. pH 8, an experimental condition known to facilitate hydrolysis of *anti*-BaPDE-derived esters via the B_{AL}1 mechanism.⁴ When benzylic and tertiary esters hydrolyze by this mechanism, oxygen from solvent water is incorporated into the alcohol(s) produced. The mechanism is unimolecular, and thus a benzylic cation is formed from *anti*-BaPDE esters as an intermediate. Attack of solvent oxygen may then be from either face of the relatively planar cation, leading to the formation of the isomeric tetrahydro BaP tetrols (i.e., 9,10-*trans* and 9,10-*cis* relative stereochemistry). By allowing benzylic esters to solvolyze in high isotopic purity H₂¹⁸O-labeled water it is possible to demonstrate their existence without actually isolating them.

Table I shows the amount of ¹⁸O incorporated into BaP tetrols under near neutral conditions of Pronase E digestion of *anti*-BaPDE-adducted human serum albumin. The *cis*-tetrol had 43% ¹⁸O incorporation, while the *trans*-tetrol incorporated 22%. The reasons for the difference are unknown, but there are several possible explanations. A related and inseparable issue which will be addressed simultaneously is the observation of less than quantitative ¹⁸O incorporation.

C-10-¹⁶O-labeled tetrols could have been present at the beginning of the digestion in H₂¹⁸O if the extraction procedures were inadequate to remove all tetrols arising from hydrolysis of *anti*-BaPDE. Such tetrols would be present in a *trans*-to-*cis* ratio of 3:1. We stress that the final 1-BuOH extract of the intact adducted albumin contained <0.1% of the added radioactive dose applied in the form of [¹⁴C]-*anti*-BaPDE to the protein. Then, if the chiral environment of serum albumin preferentially directed attack of water on the α face of the intermediate cation during hydrolysis of the ester(s) via the B_{AL}1 mechanism, a lower *trans*-to-*cis* ratio would be found in the ¹⁸O-labeled tetrols. In the combined tetrols, the *cis* isomers would exhibit a greater incorporation of ¹⁸O than would the *trans*.

It is also possible that there were indeed no C-10-¹⁶O-labeled tetrols present at the time the adducted protein was dissolved in H₂¹⁸O. In this case the only reasonable explanation for their presence at the end of the digestion is the participation of an acyl cleavage mechanism of hydrolysis. At elevated pH, as discussed below, even benzylic esters undergo hydrolysis by a hydroxide ion catalyzed acyl cleavage. However, acyl cleavage should also be considered possible at neutral pH since it has been shown that serum albumin possesses esterase activity.¹⁶

The participation of acyl cleavage by itself cannot explain the different *trans*-to-*cis* ratios for each of the two isotopically different tetrols. It is also necessary that both *r*-7,*t*-8,*t*-9,*t*-10- and *r*-7,*t*-

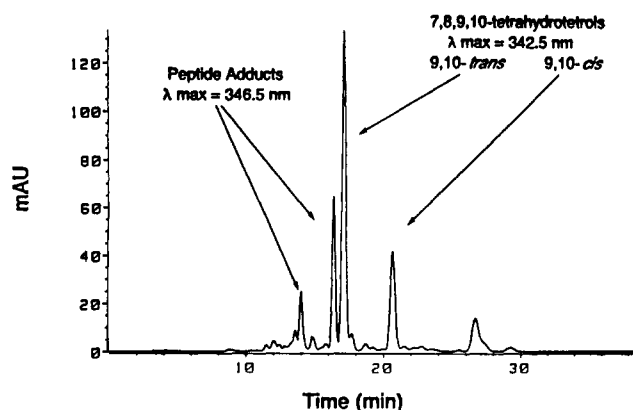


Figure 1. Reverse-phase C-18 chromatogram of the immunoaffinity chromatography bound compounds from the Pronase E digest of human serum albumin alkylated under physiological conditions with *anti*-BaPDE.

8,*t*-9,*c*-10-esters be present in the alkylated protein. Then, differential contribution of alkyl and acyl cleavage to the hydrolysis of each of the two isomeric esters, with a greater fraction of *r*-7,*t*-8,*t*-9,*t*-10-esters hydrolyzing by alkyl cleavage, could give rise to the observed isotope distributions.

Adducted human serum albumin was also digested with Pronase at pH 10 in H₂¹⁸O. Under these conditions *cis*-tetrols accounted for 21% of the total tetrols. Hydrolysis of benzylic esters under more basic conditions shifts the cleavage mode to the more familiar B_{AC}2 mechanism, where attack of solvent oxygen is at the carbonyl carbon and the stereochemistry of the ester's benzylic position is, in the absence of base-catalyzed epimerization, retained in the alcohol. The shift in mechanism is confirmed by the absence of ¹⁸O incorporation, and in the present case, as shown in Table I, no ¹⁸O was observed to be incorporated into the tetrols.

The formation of *cis*-tetrols without ¹⁸O incorporation was a surprising finding since it implies that esters with *r*-7,*t*-8,*t*-9,*t*-10 stereochemistry were present in the protein, which is apparently not the case when hemoglobin is alkylated by *anti*-BaPDE.¹⁷ This difference between the two alkylated proteins, combined with the observation that when synthetic *anti*-BaPDE C-10 esters are allowed to hydrolyze and *anti*-BaPDE-alkylated hemoglobin is digested at neutrality the *trans*- and *cis*-tetrols incorporate exactly the same fraction of ¹⁸O, lends support to the latter explanation for the differential incorporation observed when alkylated serum albumin is digested at neutrality.

Basic hydrolysis in H₂¹⁸O was performed also to determine if any of the starting epoxide was still surviving in a hydrophobic cavity of the protein, since this has been proposed to be a mechanism whereby BaPDE is transported in the circulation intact.^{8,9} Since no incorporation was observed, it may be concluded that the diol epoxide was no longer present.

Overall, the tetrols accounted for 62% of the chromatographed material from the near neutral digest. ¹⁸O was incorporated into 28% of these tetrols, proof that at least 17% of the *anti*-BaPDE adducts formed in vitro with human serum albumin are esters. Tetrols accounted for 64% of the chromatographed material from basic digestion conditions.

Histidine Adducts. Two additional components, which accounted for 22 and 8% of the chromatographed material, eluted prior to the *trans*-tetrol (Figure 1). Both compounds exhibited a pyrene S₂ absorption maximum at 346.5 nm (in the H₂O–C–H₃CN–CF₃CO₂H HPLC eluting system) and a discernible S₁ maximum at 378.5 nm. In the solvent system used for HPLC analysis, both the *cis*- and *trans*-BaP tetrols have an S₂ maximum at 342.5 nm and an almost indiscernible S₁ maximum at 376.5 nm. We, and others have noted variations of the red shift in the S₂ 0–0 transition band from that of the tetrols which is dependent on the nature of the heteroatom attached to C-10 of the 7,8,9-

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Table II. Collision-Induced Dissociation Mass Spectral Data from Peracetylated *N*^ε-Histidinyl Benzo[*a*]pyrene *anti*-Diol Epoxide Adducts

ion	nominal <i>m/z</i>	relative abundances normalized to <i>m/z</i> 285			
		BaPDE-His	minor peptide adduct (BaPDE-His-Pro)	major peptide adduct (BaPDE-His-Pro-Tyr)	synthetic BaPDE-His-Pro-Tyr
P	70			3.5	2.8
H	110	4.4	4.5	6.4	3.3
a ₁	152		2.9	4.4	3.3
b ₁	180		4.7	3.6	5.4
a	285	100	100	100	100
a	327	43.7	9.9	35.9	75.1
a	369	7.6	5.7	2.6	3.0
		5.6	9.3	5.0	4.0
		(<i>m/z</i> 198)	(<i>m/z</i> 295)	(<i>m/z</i> 500)	(<i>m/z</i> 500)
			4.2		
			(<i>m/z</i> 581)		
			(M-BaPDE) + H		
			(a ₁ + peracetylated BaPDE) + H		

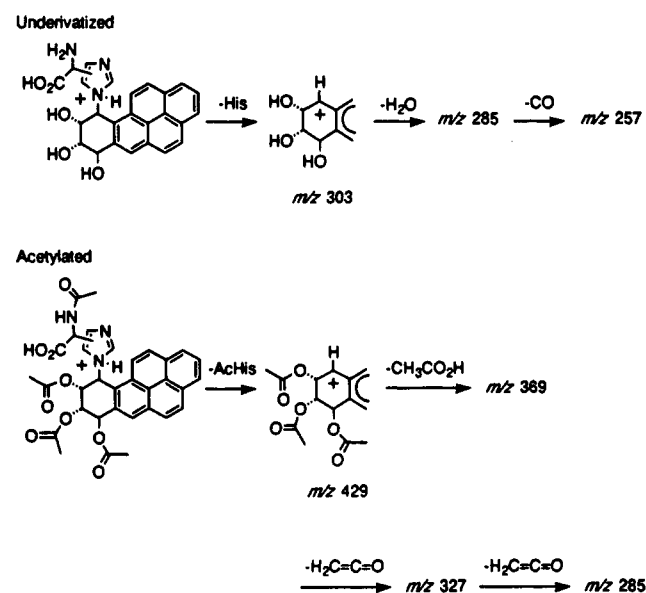
^a See text and Scheme I for descriptions of these ions.

trihydroxy-7,8,9,10-tetrahydro BaP moiety.^{4,18} The adducts we observed in the serum albumin digest have an S₂ maximum different from the S₂ of tetrols (342.5 nm) or adducts with alkylamino (344.5 nm) or alkylthio (348.5) C-10 substituents.⁴

Amino acid compositional analysis of the major intact adduct revealed the presence of equimolar amounts of histidine, proline, and tyrosine. On the assumption that these three amino acids constitute completely the peptide portion of the adduct, the sequence of human serum albumin was searched for all subsequences satisfying this compositional requirement. Only one was found, namely His(146)-Pro(147)-Tyr(148). The adduct was thus presumed to be formed by alkylation of either His(146) or Tyr(148). Edman sequencing analysis of this major stable adduct yielded a sequence of *N*-unknown-Pro-Tyr-C, consistent with the premise that alkylation had taken place on the histidine residue.

Amino acid analysis of the chromatographic fraction containing the minor intact adduct revealed the presence of a large background of amino acids as well as a significant amount of histidine. The most predominant amino acids in this fraction were Ser, Pro, Gly, and Leu. Only the serine side chain is nucleophilic in this group. Alkyl ethers of *anti*-BaPDE (i.e., trans and cis C-10 ethoxy ethers) were synthesized, and their UV spectra were indistinguishable from those of the tetrols. Hence, the minor *anti*-BaPDE-peptide was likely not an alkyl ether. Edman sequencing of the peptides within this chromatographic fraction yielded results which suggested that the minor adduct consisted of *N*-unknown-Pro-(Asp)-(Tyr)-C, although the background was too high for confidence in assigning the latter two amino acids to the peptide. There is only one subsequence in human serum albumin that contains these four amino acids: His(338)-Pro(339)-(Asp(340))-(Tyr(341)). The UV spectrum of this fraction was identical with that of the major adduct chromatographic fraction. Both the imidazole and carboxylate side chains are nucleophilic in this sequence. Carboxylic esters of *anti*-BaPDE do not survive the enzymatic reaction conditions we used to generate the peptides from the alkylated albumin. The only adduct which could satisfy both the physical and spectroscopic requirements noted above would be a modified imidazole. Hence, the His(338) residue could be the modified amino acid. Another subsequence of the protein, His(440)-Pro(441), could conceivably have yielded peptide sequencing data consistent with alkylation of its imidazole side chain. A final possibility is that the minor adduct was a fragment, consisting of His(146)-Pro(147), of the major adduct.

Positive ion FAB-MS of underivatized adducts yielded precursor ions abundant enough for CID analysis only for the synthetic histidine-*anti*-BaPDE adducts (vide infra). The M + H ion abundance for all of the adducts increased markedly when the samples were peracetylated with acetic anhydride/pyridine. CID spectra were then obtained for peracetylated adducts of histidine and di- and tripeptides, both synthetic and protein-derived. The

Scheme I. Proposed Collision-Induced Dissociation Reaction Pathways of the Underivatized and Acetylated *N*^{im}-Histidine-*anti*-BaPDE Adducts

CID spectra of these peracetylated compounds are dominated by ions of *m/z* 369, 327, and 285, representing losses of acetic acid, acetic acid + ketene, and acetic acid + 2 mol of ketene, respectively, from the BaPDE-derived moiety (Scheme I). Fragment ions produced from the peptide portion of the adducts were few and were of low abundance. Peptide ion fragment series with the BaPDE residue still attached were observed in some, but not all cases (Table II). Each acetylated peptide adduct yielded spectra which were consistent with the sequencing data.

Synthetic Adducts for Structure Confirmation. For comparison of electronic absorption and emission characteristics, synthetic imidazole ring-substituted histidine adducts were prepared. Each of the two imidazole nitrogens was alkylated by reaction of histidine with *anti*-BaPDE at pH 7.5, and the products were purified by C-18 HPLC. The ratio of the products was essentially 1:1. The UV absorption spectra of these two compounds were identical with each other as well as to the spectrum of the serum albumin adducts.

The *N*^ε- and *N*^δ-adduct isomers were distinguished by a previously described fluorescence method.¹⁵ Each histidine adduct was reacted with fluorescamine to derivatize their respective α-amino nitrogens. After treatment with boiling aqueous HCl, only the fluorescamine derivative of the later eluting adduct formed a product with altered fluorescence (e.g., fluorescence which was different from the pyrene fluorescence), which indicated the formation of a new electronic structure. Since the formation of

a new fluorescent species by this procedure requires a free π -nitrogen for an intramolecular reaction,¹⁵ the position of alkylation was assigned to N τ . The earlier eluting synthetic histidine-*anti*-BaPDE adduct did not form a species with the new fluorescence and was thus determined to be N τ -substituted.

Unlike the enzymatically stable peptide adducts, both histidine adducts were amenable to FAB-MS analysis without derivatization, but peracetylation did yield samples which gave much stronger signals in the FAB-MS experiments. The fragmentation seen in the CID spectrum of the peracetylated N τ -histidine-*anti*-BaPDE adduct was, as with the peptide adducts isolated after proteolysis of the BaPDE-serum albumin reaction, dominated by ions resulting from the breakdown of the a-ring by sequential loss of ketene (m/z 369, 327, 285). The fragmentation of the peracetylated N τ -histidine-*anti*-BaPDE adduct differed from that of the N τ -adduct only in the relative intensities of the fragments formed (Table II).

It was possible to gain information on both the sequence of the peptide and the location of the *anti*-BaPDE derived residue for some of the peptide adducts. In the CID spectrum of peracetylated *anti*-BaPDE-N τ -His-Pro, the minor peptide adduct from the Pronase digested protein, the ion corresponding to the protonated, peracetylated peptide after loss of the *anti*-BaPDE derived moiety was noted (m/z 295), as was the a_1 ion covalently modified by *anti*-BaPDE (m/z 581). The presence of this latter ion shows clearly that the site of adduction on this peptide is the imidazole side chain of histidine.

Synthetic His-Pro-Tyr was prepared by FMOC/BOP chemistry and alkylated with *anti*-BaPDE at pH 6.5. The yield of adducts from this alkylation reaction was very low, even when using >100-fold molar excess of the tripeptide. Like histidine, N-His-Pro-Tyr-C formed two major adducts when reacted with *anti*-BaPDE, both with UV spectra identical with the spectra of adducts isolated from Pronase-digested, *anti*-BaPDE alkylated human serum albumin. The earlier eluting synthetic peak had a retention time intermediate to those of the adducts from albumin, while the later coeluted with the major adduct. The later eluting component from the synthetic His-Pro-Tyr + *anti*-BaPDE reaction was positive for the fluorescamine/acid/heat reaction in the same manner as the later eluting synthetic histidine-*anti*-BaPDE adduct, indicating that this component was τ -nitrogen alkylated as well. The earlier eluting compound did not yield a fluorescent species under these reaction conditions, indicating that its π -imidazolyl nitrogen was alkylated. The later eluting fraction was collected, lyophilized, and analyzed by HPLC, followed by FAB mass spectrometry of its peracetyl derivative. Its CID spectrum was identical with that obtained from the major peptide adduct, with the same major fragment ions and fragmentation patterns seen with the N τ -histidine-*anti*-BaPDE and *anti*-BaPDE-N τ -His-Pro adducts. Synthetic *anti*-BaPDE-N τ -His-Pro-Tyr and the major albumin-derived adduct cochromatographed as a single peak when analyzed by coinjection on C-18 HPLC.

Conclusions

The data in the present study revealed that imidazole and carboxylate side chains in serum albumin are alkylated in vitro by *anti*-BaPDE. His(146) was clearly the most reactive histidine. The data are also compatible with alkylation of His(338) and/or His(440) but neither confirmed nor rejected the possibility. No evidence was obtained for the alkylation of any other histidine residue. His(146) and His(338) correspond to the two histidine residues in bovine serum albumin (145 and 336) which are alkylated by dansylaziridine.¹⁹ Notably, bovine His(145) is far more reactive toward dansylaziridine than bovine His(336).

Both His(146) and His(338) are known to be part of a site that is involved in the noncovalent binding of functionalized, bulky, aromatic molecules, such as benzodiazepines²⁰ and acetyl-L-

tryptophan.²¹ Both of these residues are adjacent to prolines, and they are likely at bends of the protein which are in close contact in the protein's tertiary structure.²²

Incorporation of ¹⁸O into BaP tetrahydrotetrols released by enzymatic hydrolysis of the adducted protein was used to prove that carboxylic esters are a major product from the reaction of *anti*-BaPDE with human serum albumin in vitro. Even though the esters are likely stable in the intact protein, this approach was necessary because the esters are too unstable to permit the isolation of ester-bearing peptide fragments. Consequently, the carboxylate-containing amino acid residues involved in ester formation could not be identified. It is in principle possible to determine the reacting residue by conversion of its ester to a more stable amide, as we have shown with *anti*-BaPDE-alkylated hemoglobin,³ but it is not clear if the requisite subsequent and protracted steps of disulfide reduction and selective proteolysis can be effected with *anti*-BaPDE-alkylated human serum albumin.

Previous reports concerning human serum albumin treated with *anti*-BaPDE state that small amounts of *stable* adducts are formed: 2% of the dose applied to the protein at pH 4.9, 7% at pH 6, 10% at pH 7.2, and 11% at pH 8.1.^{7,8} This pH dependence is consistent with the alkylation of histidine's imidazole nitrogen, which has a pK' in the free amino acid, of 5.97. The results from this and a related study¹¹ indicate that the imidazole of (a) histidine(s) is indeed modified by *anti*-BaPDE and that in fact it is the τ -nitrogen which is alkylated. Human serum albumin carboxylic esters of *anti*-BaPDE, unlike those in human hemoglobin, have not yet been proven to be stable in vivo. As with *anti*-BaPDE-human hemoglobin esters, the corresponding albumin esters are likely stable, i.e., the ester bond is protected from water, as long as the protein retains its tertiary structure.⁴ Previously reported works on the interaction of this protein with *anti*-BaPDE^{8,9} were not performed in a manner which would have accounted for adducts which were hydrolytically unstable when the protein tertiary structure was disturbed.

While the underivatized peptide adducts were difficult to ionize by fast atom bombardment, their peracetylated derivatives gave protonated molecular ions of high abundance. The synthetic and protein-derived adducts then yielded identical collision induced dissociation mass spectral fragmentation data.

In summary, we have determined that two types of adducts are formed when *anti*-BaPDE alkylates human serum albumin. One is a carboxylic ester, which is relatively unstable and eludes isolation as a proteolytic peptide fragment. Consequently, the site-specificity of its formation remains unknown. The other, a stable N τ -substituted histidine, is formed predominately if not exclusively by one histidine residue, namely His(146). Highly specific interactions with ligands is characteristic of serum albumin, and it appears that the binding of *anti*-BaPDE to human serum albumin is no exception. This enzymatically and hydrolytically stable adduct may prove useful both as a dosimeter for quantitation of active BaP metabolite reaching the circulatory system as well as a fluorescent probe of the three-dimensional structure of the protein.

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