Isolation and Characterization of the Major Fluoranthene-Hemoglobin Adducts Formed in Vivo in the Rat

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ABSTRACT

The binding of fluoranthene (FA) to hemoglobin was studied both in vitro and in vivo in the rat. The in vitro binding of microsomal activated FA to rat hemoglobin appeared to involve the fluoranthene 2,3-dihydrodiol-1,10b-oxepoxides. Three classes of hemoglobin adducts were observed in rats chronically administered FA in the diet. Based on high pressure liquid chromatography retention times, UV and mass spectral evidence, and behavior upon cis-diol affinity chromatography, the major class of globin adducts formed in vitro was demonstrated to result from binding of syn and anti isomers of FA 2,3-dihydrodiol-1,10b-epoxides to β-cysteine-125 of rat hemoglobin. These adducts represented at least 41% of the total binding to globin. A minor class of adducts (12% of the total binding) appeared to involve the binding of an unidentified FA metabolite to the same cysteine residue of the protein. A substantial portion of FA binding to rat hemoglobin in vivo (29%) involved metabolic pathways which were not duplicated by simple in vitro systems. That portion of the binding to globin has not been characterized.

INTRODUCTION

FA is a polycyclic aromatic hydrocarbon of widespread occurrence and significant biological activity. It is a combustion product formed by the burning of fossil fuels (1-3), the pyrolysis of tobacco (4), and by industrial processes such as coke production (5), coal gasification (6), and iron and steel founding (7). It is a potent mutagen toward bacteria (8) and human cells (9) and is both a carcinogen (10) and a cocarcinogen (11) in rodents. As an environmental contaminant, FA is almost always found in excess over benzo(a)pyrene, the most frequently studied polycyclic aromatic hydrocarbon.

The use of protein adducts as dose monitors for carcinogens is important. The method of using covalent protein adducts of polycyclic aromatic hydrocarbons formed in vivo has been developed by J.M. Osterman-Golkar and co-workers (12-14). They found that the adducts formed in vivo have a molar ratio of protein to adducts approximately 1:1 (12, 13). Each adduct is a single molecular species of the original polycyclic aromatic hydrocarbon. The potential for extending this approach to other types of carcinogens was recognized (15) early in its development. Additional types of adducts have been characterized, including 3-mercaptopropionic acid adducts (16) formed with 3-MPA. A procedure for the isolation and characterization of tumor protein adducts was developed and is summarized in a review article (17). It is important to be able to isolate and characterize tumor protein adducts for they are the dose monitor for the carcinogen.

MATERIALS AND METHODS

Chemicals. [8-3H]FA (23.7 Ci/mmol) was custom synthesized by Midwest Research Institute (Kansas City, MO) and purified before use by chromatography on silica gel with the use of hexane: dichloromethane (3:2). HPLC analysis of the purified material showed greater than 95% radiochemical purity. [3H]Fluoranthene 2,3-dihydrodiol-1,10b-epoxides (anti isomer, 20.4 mCi/mmol; syn isomer, 10.3 mCi/mmol) were the products of previously reported syntheses (18). Pepsin was obtained from Sigma Chemical Co. (St. Louis, MO) and Pronase from Calbiochem-Behring (La Jolla, CA). Affi-Gel 601 was from Bio-Rad (Richmond, CA). Sephadex LH-20 was purchased from Pharmacia (Piscataway, NJ). Aroclor 1254-induced rat liver microsomes were obtained from Litton-Bionetics (Kensington, MD).

In Vivo Modification of Hemoglobin. Hemoglobin modification with fluoranthene 2,3-dihydrodiol-1,10b-epoxides was carried out in the following manner. Freshly isolated, washed, rat erythrocytes were suspended in phosphate-buffered saline, pH 7.5, up to the original blood volume. The suspension was added to a vial containing the appropriate isomer of FA diol-epoxide from which the solvent (tetrahydrofuran) had been evaporated. The molar ratio of hemoglobin to FA diol-epoxide was 20:1, based on a concentration of hemoglobin in rat blood of 15.6 g/100 ml. The mixture was incubated at 37°C for 3 days (owing to the prolonged stability of FA diol-epoxides at physiological pH). At the end of the incubation, the RBC were recovered by centrifugation, washed 3 times with normal saline, and lysed with 3-4 volumes of 0.7 M phosphate buffer, pH 5.5-6, plus one-tenth volume of toluene. After centrifugation to remove the cellular debris, the resulting hemoglobin solution was dialyzed exhaustively against distilled, deionized water prior to the isolation of globin.

For microsomal activation of FA binding to hemoglobin, freshly isolated, washed rat erythrocytes (2 µmol of hemoglobin based on the concentration in blood) were added to a 5-ml incubation mixture containing 25 µm FA (8-3H)FA, 231 mCi/mmol), 0.5 mg/ml microsomal protein (Aroclor 1254-induced rat liver microsomes), 0.7 mm NADP+, 5.0 mM glucose 6-phosphate, 3 mM magnesium chloride, 0.5 µM glucose-6-phosphate dehydrogenase, and incubated at 37°C for 2.5 h. At the end of the incubation period, the RBC were recovered by centrifugation, washed with normal saline, and lysed in the usual manner. After centrifugation to remove cellular debris and microsomal protein, the hemoglobin solution was dialyzed against distilled, deionized water prior to the isolation of globin.

Animals and Dosing. Dosing protocols were designed primarily for the purpose of maximizing adduct accumulation and not for studying quantitative aspects of dosimetry. Twenty-one male Sprague-Dawley rats (Charles River), average weight of 99 g, were placed on a complete, agar-based diet containing FA. The diet composition (per batch) was casein, 18 g; sucrose, 20.5 g; dextrose, 24.8 g; dextrin, 24.8 g; corn oil, 5 g; mineral mix, 5 g; standard vitamin mix, 2 g; standard choline chloride solution, 15 ml; standard vitamin B12 solution, 5 ml; agar, 50 g; water, 1 liter. To prepare the contaminated diet, FA was first dissolved in corn oil; the dry ingredients were added and thoroughly blended with the oil; the agar and vitamins were added with stirring to form a homogeneous mixture which was cooled and cut into pieces for feeding.

The animals were fed ad libitum, and the weight of diet consumed was recorded to estimate the intake of FA. The level of fluoranthene in the diet was 250 ppm for the first 2 weeks and 500 ppm for the remaining 4 weeks of the study. Average daily intake of FA was estimated at 180 mg/kg body weight.

Six rats were given radiolabeled FA (8-3HFA; 23.7 mCi/mmol) as a tracer in addition to the FA contained in the diet. Over the first 19 days of the study, each of the six rats received an i.p. injection of 36 µCi of radiolabeled FA every 2 to 3 days, for a total of seven injections. An eighth dose of 218 µCi was administered at 3 weeks and the six rats were sacrificed 2 days later. After receiving the last i.p. dose, these

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3 The abbreviations used are: FA, fluoranthene; FAB, fast atom bombardment; MS, mass spectrometer; E, electric sector; B, magnetic sector; HPLC, high performance liquid chromatography; 3-MPA, 3-mercaptopropionic acid.

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animals continued to eat uncontaminated diet. The six rats were sacrificed early to ensure that sufficient protein-bound activity for subsequent use as a tracer was obtained. Of the remaining animals, 7 were sacrificed after 5 weeks on the diet and 8 were sacrificed after 6 weeks. Blood was obtained by cardiac puncture under ether anesthesia and hemoglobin was prepared from the erythrocytes as previously described.

Preparation and Digestion of Globin. Globin was prepared by extraction of hemoglobin at pH 1.5–2 with 2 volumes of ice-cold methanol. The globin remained in solution in the aqueous phase and was dialyzed against distilled, deionized water. Seventy % of the total radioactivity in hemoglobin from FA-treated rats was bound to globin.

The globin solutions were adjusted to pH 2 with HCl and the protein was digested overnight at 37°C with pepsin (0.1 mg/mg hemoglobin). Following the digestion, concentrated phosphate-buffered saline (10× phosphate-buffered saline) was added to a final concentration of 1× phosphate-buffered saline and the pH was adjusted to 7.5–8. Isopropyl alcohol (5%, v/v) was added to prevent bacterial growth. The mixture was digested overnight at 37°C with Pronase (0.1 mg/mg hemoglobin). The digests were centrifuged to remove precipitated material prior to further analysis.

Chromatography. Digests of globin from FA-treated rats, or of globin modified in vitro with FA derivatives, were chromatographed on a Whatman M9 ODS-3 preparative reverse-phase HPLC column. The weak solvent was 0.05 M ammonium formate buffer, pH 6.4, containing 10% methanol, and the strong solvent was methanol. The gradient program was weak solvent for the first 10 min, followed by a linear gradient to 100% methanol in 40 min. The flow rate was 2.5 ml/min. One-min fractions were collected for the determination of the profile of radioactivity in the digests.

Enzymatic digests of in vitro-modified globin were applied directly to a column of Affi-Gel 601 boronic acid gel for cis-diol affinity chromatography (7.5 cm x 1 cm inside diameter) equilibrated with 0.05 M ammonium formate buffer, pH 8.5. Bound material was eluted with 0.9% formic acid, pH 3, and immediately neutralized with 2 N ammonium hydroxide. Enzymatic digests of globin from FA-treated animals were prefractionated as described in the next section before chromatography on Affi-Gel 601 under the same conditions.

Isolation of the Major FA-Globin Adducts Formed in Vivo. The procedure is outlined in Fig. 1. The designation of adducts as S, A, or U is intended to indicate whether they were formed by syn or anti FA diol-epoxide or by an unknown metabolite.

The digest of globin isolated from FA-treated rats was applied to a column of Whatman Partisil 53 preparative C18 media packed in water. The bulk of unmodified peptides was removed by continued elution with water and radioactivity in the digest was eluted with 35% methanol in water, followed by 50% methanol in water. The radioactive material obtained from this column was chromatographed under isocratic conditions on Sephadex LH-20 (column, 1.5 cm inside diameter x 48 cm; flow rate, 0.5 ml/min), using 0.05 M ammonium formate, pH 6.4, containing 5% methanol. Eighteen % of the total radioactivity eluted as a broad tailing peak, beginning at the void volume. Two major fractions containing radioactivity were obtained. LH-20 fraction I eluted between 140 and 180 min, and contained 53% of the total radioactivity bound to globin. LH-20 fraction II eluted between 180 and 220 min and contained 29% of the total radioactivity bound to globin.

LH-20 fraction I was further purified by chromatography on the Whatman M9 ODS-3 column under the conditions described above. Radioactivity eluted as one peak between 24 and 28 min. This material was then fractionated by Affi-Gel 601 affinity chromatography to separate adducts having cis-1,2 hydroxy groups. The unbound material from the phenyl boronic acid column was chromatographed on a Whatman Partisil 5 preparative reverse-phase HPLC column, using 0.05 M ammonium formate buffer containing 10% methanol as weak solvent (A) and methanol as strong solvent (B). A concave gradient was used (curve 8 on the Waters automated gradient controller, Milford, MA) by using starting conditions of 10% solvent B and reaching 30% solvent B in 40 min; the flow rate was 0.75 ml/min. Three adduct peaks were observed: S1 and S2 (retention times 21.5 and 23.5 min, respectively) and U (retention time, 27.5 min). The adducts were collected, chromatographed once more under the same conditions, and then desalted by chromatography on the same column, using isocratic elution with 10% methanol in water.

Material which bound to phenyl boronic acid was chromatographed on the Whatman P5 ODS-3 column as just described. Two peaks were observed: A1 had a retention time of 24.8 min and A2 had a retention time of 25.8 min. The adducts were collected and desalted.

The radioactive material from LH-20 fraction II was also chromatographed on the Whatman ODS-3 column under the conditions described above. The radioactive material eluted as a single peak between 29 and 30 min. This was the same retention time as peak II, initially observed by semipreparative reverse-phase HPLC. Digests obtained from globin modified in vitro with fluoranthene derivatives did not display radioactivity with a similar retention time. Thus the adducts in LH-20 fraction II were formed only in vivo. Characterization of these adducts in progress, while the adducts found in LH-20 fraction I form the principal subject of this paper.

Spectral Analyses. UV spectra were obtained with a Hewlett Packard 8450 spectrophotometer. Tandem FAB mass spectra were acquired with a four sector mass spectrometer (JEOL HX110/HX110) of EB/EB configuration. Less than 1 nmol of adduct was needed for the mass spectral analyses.

Amino Acid Analyses. Adduct samples (approximately 3 nmol) were dissolved in 1.0 ml of 6 N HCl, sealed in evacuated glass tubes, and heated at 110°C for 18 h. After hydrolysis, the samples were evaporated to dryness, redissolved in lithium citrate buffer, pH 2.2, and analyzed with a Durrum Dionex amino acid analyzer.

RESULTS

Comparison of Fluoranthene Binding to Hemoglobin in Vivo and in Vitro

The major pathway for metabolic activation of FA to mutagens in vitro involves formation of the trans-FA 2,3-dihydrodiol and subsequent oxidation of this diol to syn and anti isomers of FA 2,3-dihydrodiol-1,10b-epoxides (FA diol-epoxides) (21, 22). The structures of FA and its diol-epoxides are shown in Fig. 2.
The role of these compounds in hemoglobin binding was assessed by incubating rat erythrocytes with either radiolabeled FA in the presence of rat liver microsomes or with radiolabeled syn or anti isomers of FA diol-epoxide. Globin prepared from the RBC was digested enzymatically and the digests were chromatographed by semipreparative HPLC. The profiles of radioactivity observed in these digests were compared with similar digests of globin isolated from rats administered FA (Fig. 3).

Fig. 3a shows the profile of radioactivity in globin digests derived from rats which had received FA chronically in the diet. Two peaks of radioactivity, corresponding to 53 and 29% of the total binding to globin, were observed (Fig. 3a, Peaks I and II). The remaining 18% of the radioactivity eluted as a broad, tailing peak. A similar profile of radioactivity on HPLC was observed in globin digests obtained from rats given a single low dose of radiolabeled FA (data not shown). Analysis of LH-20 fraction I and II on the ODS-3 column revealed that they corresponded to peaks I and II, respectively.

Digests of globin modified in vitro with microsomally activated FA (Fig. 3b) or the anti or syn FA diol-epoxides (Fig. 3, c and d) each displayed a single peak of radioactivity with a retention time the same as that of peak I, the major radioactive peak observed in digests of globin from rats administered FA. This indicated that peak I would contain adducts of the FA diol-epoxides and that adducts eluting with the retention time of peak II were not diol-epoxide adducts.

Characterization of the Major FA-Globin Adducts Formed in Vivo

Chromatographic Behavior and UV Spectra. Adducts S1 and S2 represented 29% of the total radioactivity bound to globin. These adducts did not complex with phenyl boronic acid. When analyzed by analytical reverse-phase HPLC (Fig. 4), they had the same retention time as the syn FA diol-epoxide adducts prepared in vitro (data not shown). Adducts A1 and A2 were obtained from the fraction of the globin digest which bound to phenyl boronic acid. They represented 12% of the total radioactivity bound to globin, and had the same retention times on analytical reverse-phase HPLC as anti FA diol-epoxide adducts prepared in vitro. Their identification as anti FA diol-epoxide adducts is supported by the binding to phenyl boronic acid (23-25). Adduct peak U was present in the unbound fraction from the phenyl boronic acid column and represented 12% of the total radioactivity bound to globin. Its retention time by analytical reverse-phase HPLC was distinct from that of the FA diol-epoxide adducts.

The UV spectra support the conclusion that the S and A adducts originated from syn and anti FA diol-epoxides, respectively. By comparison with the spectra of model adducts formed by reaction of the FA diol-epoxides with 3-MPA they also suggest that S and A are formed by reaction with cysteine. Adducts S1 and S2 each had a spectrum which displayed one maximum at 262 nm as did the syn FA diol-epoxide-3-MPA adduct spectrum. In contrast, the spectrum of each of the 2 tetrols derived from syn FA diol-epoxide consisted of 3 maxima at 229, 237, and 275 nm. Similarly, the spectra of adducts A1, A2, and the anti FA diol-epoxide-3-MPA adduct all displayed one maximum at 267 nm, whereas the spectrum of the single anti FA diol-epoxide-derived tetrol was composed of 3 maxima at 231, 239, and 277 nm.

Adduct peak U displayed an absorbance maximum at 262 nm, suggesting a similar chromophore to that of the syn diol-epoxide adducts; however, based on its HPLC retention time,
Chemistries, also had identical molecular weights, 770, and are which were formed by FA diol-epoxides of differing stereo anti FA diol-epoxide) both had a molecular weight of 642. They therefore was assumed to consist of two components not resolved thus was assumed to consist of two components not resolved upon fractionation. Tandem MS spectra of U1 and U2 displayed the same ions, 391 and 519, but were produced by loss of 298 from the molecular ions 689 and 817, respectively. The neutral fragment 252 is almost certainly the fluoranthene moiety since the fragment mass corresponds to the empirical formula C_{13}H_{25}O_{9}, as expected for a structure derived from the diol-epoxides by epoxide ring opening. Presumably 298 is also FA derived, but it is not obvious what it might be. Assuming that the fragment ions 391 and 519 correspond to peptides, the difference between them, 128 atomic mass units, is significant because it is equal to the molecular weight of either glutamine or lysine. It is thus highly likely that one peptide forms the S1, A1, U1 series adducts and another peptide forms the S2, A2, U2 series adducts, and that the two peptides are identical except for the absence or presence of a terminal amino acid.

The only sequence of amino acids in rat hemoglobin which can yield a peptide of molecular weight of 518 occurs in the major β chain (28). That sequence is Thr-Pro-Cys-Ala-Gln. As expected from the mass difference between the two major peptide ions, this sequence does have a terminal amino acid with molecular weight of 128, namely glutamine.

Confirmation of the inferred sequence for the binding site for fluoranthene diol-epoxides was obtained from further analysis of the collision-induced decomposition spectra. A major class of fragment ions observed in the MS-MS spectra of peptides results from cleavage of the peptide bonds, starting from either the amino or carboxy terminus. Sequence information is obtained from series of ions which differ by the residual mass of each consecutive amino acid in the peptide (26, 27). Charge retention at the amino terminal portion gives rise to so-called “b series” ions, while charge retention on the carboxyl terminus gives ions of the y series (29).

In the present case, no sequence ions derived from the molecular ions were observed. However, there were clear sequence ions derived from the major fragment ion produced by loss of 252 (A1 and S2) or by loss of 298 (U1 and U2). These ions provided a complete determination of the sequences as well as confirmation that the major fragment ions were peptides composed solely of unmodified amino acids.

The peptide fragment of molecular weight 518 displayed a b series of ions derived from initial loss of either glutamine or lysine from the carboxy terminus, followed by the loss of alanine and cysteine. The identification of the carboxy-terminal amino acid as glutamine by inspection of the sequence of rat hemoglobin is confirmed by the observation of a M + H-72 ion [loss of the side chain of glutamine (27)] in the spectra of S2 and U2, but not in the spectra of A1 and U1. A single y series ion was observed which corresponded to the loss of threonine from the amino terminus of the peptides. A characteristic imminium ion at mass 70 (26) showed that proline was also present in the peptides. Hence, for the adducts of higher molecular weight, the peptide sequence revealed was Thr-Pro-Cys-Ala-Gln.

In the adducts of lower molecular weight, b series ions from the peptide of molecular weight 390 resulting from loss of alanine and cysteine from the carboxy terminus, a y series ion showing loss of threonine from the amino terminus, and a proline imminium ion were observed. The sequence derived in this case was Thr-Pro-Cys-Ala.

Amino acid analysis was performed to corroborate the composition implied by tandem FAB MS (Table 1). Although some impurities were observed, the analysis confirmed the presence

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**FA-HEMOGLOBIN ADDUCTS**

This peak was distinct from adducts of the FA diol-epoxides.

**Peptide Sequencing of Fluoranthene-Hemoglobin Adducts by Tandem FAB Mass Spectrometry.** Further information on the structures of the adducts was obtained by tandem FAB mass spectrometry (26, 27). The advantage of this technique is that sequence information can be obtained on adducted or posttranslationally modified peptides or mixtures of peptides. The principle of the method is as follows. Analysis by the first mass spectrometer provides information on the molecular weight of the sample components, which are detected as protonated molecular ions. The ions of interest produced by the first instrument are then selectively introduced into a collision chamber, where they undergo fragmentation. The fragment ions are separated and detected by the second instrument, and from the resulting mass spectrum, sequence information can be derived.

A molecular ion was obtained for each of the isolated adducts by two-sector MS. Adduct U displayed two prominent ions and thus was assumed to consist of two components not resolved by chromatography, which are designated U1 and U2. Adducts A1, S2, U1, and U2 were analyzed by tandem MS as well.

Adducts S1 (from the syn FA diol-epoxide) and A1 (from the anti FA diol-epoxide) both had a molecular weight of 642. They were known to differ in the stereochemistry of the FA diol-epoxide from which they were formed. The identity of their molecular weights suggests that their peptide residues are identical and that they are chromatographically separable because of stereochemical differences. Similarly, adducts S2 and A2, which were formed by FA diol-epoxides of differing stereochemistries, also had identical molecular weights, 770, and are likely to have identical peptide residues.

The tandem MS spectra of A1 and S2 are dominated by a neutral loss of 252 mass units to yield ions of mass 391 and 519, respectively. Fig. 5 presents the spectrum of S2 to illustrate this and other observed fragmentations. Tandem MS spectra of U1 and U2 displayed the same ions, 391 and 519, but were produced by loss of 298 from the molecular ions 689 and 817, respectively. The neutral fragment 252 is almost certainly the fluoranthene moiety since the fragment mass corresponds to the empirical formula C_{13}H_{25}O_{9}, as expected for a structure derived from the diol-epoxides by epoxide ring opening. Presumably 298 is also FA derived, but it is not obvious what it might be. Assuming that the fragment ions 391 and 519 correspond to peptides, the difference between them, 128 atomic mass units, is significant because it is equal to the molecular weight of either glutamine or lysine. It is thus highly likely that one peptide forms the S1, A1, U1 series adducts and another peptide forms the S2, A2, U2 series adducts, and that the two peptides are identical except for the absence or presence of a terminal amino acid.

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**Fig. 4.** Analytical reverse-phase HPLC separation of fluoranthene-hemoglobin adducts isolated from LH-20 fraction I.
of near stoichiometric amounts of threonine, proline, alanine, and glutamine in the appropriate adducts.

**Stereochemistry of Epoxide Ring Opening.** Adducts A1 and A2, which are formed by the *anti* FA diol-epoxide, bound to immobilized phenyl boronic acid. This is consistent only with attack by cysteine at the C-10b position, for if the epoxide were opened at C-1, then the only vicinal diol would be at C-2, C-3, and these hydroxyls are known to be *trans*. Whether the epoxide opening occurs *cis* or *trans* cannot be inferred. Adducts S1 and S2 did not bind, and thus may be assumed not to have vicinal diols which are *cis* to each other. This observation does not indicate which of the possible ring openings occurred, but it also does not rule out attack at C-10b, which is the expected site from the observations made on adducts A1 and A2.

**DISCUSSION**

The binding of FA to hemoglobin was studied both *in vitro* and in rats chronically administered FA in the diet. Adducts formed *in vitro* from microsomal activated FA and rat hemoglobin appeared to result solely from the binding of the FA diol-epoxides. In contrast, three distinct classes of adducts were observed in treated rats. The major class of adducts, which diol-epoxides. In contrast, three distinct classes of adducts were observed in treated rats. The major class of adducts, which...
radioactivity bound to hemoglobin was very similar to the amounts of FA dial-epoxides which would have been formed under the conditions of the experiment (data not shown), suggesting that rat hemoglobin was a very efficient trap for these electrophiles.

FA dial-epoxides reacted specifically at β-cysteine-125 of rat hemoglobin, a residue which has been identified as a highly reactive nucleophilic site on the protein (30). Since the amino acid residue at this position of human hemoglobin is proline, FA dial-epoxide adducts to human hemoglobin will involve a different target nucleophile. We found no evidence for reaction at β-cysteine-93, which is a reactive thiol group common to all mammalian hemoglobins. It may simply be that the rate of reaction with cysteine-125 is so much greater than the rate of reaction with cysteine-93 that the latter is unapparent. Human hemoglobin can be modified in vitro by the FA dial-epoxides, and experiments aimed at identifying the site of binding are currently under way.

Our results underscore the need for caution in the choice of appropriate laboratory models for the study of carcinogen-protein interactions, particularly with the view toward dosimetry in humans. We believe that the approach of structure elucidation is an important first step in understanding the factors which influence protein binding of carcinogens. In addition, the information gleaned from such studies could assist in the development of methods for detecting carcinogen-protein adducts in human populations.

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