Purification of a Benzo[*a*]pyrene Binding Protein by Affinity Chromatography and Photoaffinity Labeling†

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ABSTRACT: Binding proteins for the polycyclic aromatic hydrocarbon carcinogen benzo[*a*]pyrene (B[*a*]P) have been purified from C57B1/6J mouse liver. Following affinity chromatography on aminopyrene–Sepharose, a single polypeptide of 29 000 daltons was isolated. The photolabile compound 1-azidopyrene was developed as a photoaffinity labeling agent to identify the protein during its purification. 1-Azidopyrene was found to be a competitive inhibitor of [^{3}H]B[*a*]P binding. Affinity labeling studies with [^{3}H]-1-azidopyrene in unfractionated cytosol, and in purified preparations, yielded a single covalently labeled protein of 29 000 daltons. The formation of this labeled species was blocked by preincubation with excess unlabeled B[*a*]P. A native molecular weight of 30 000 was estimated by gel filtration chromatography of [^{3}H]B[*a*]P- and [^{3}H]-1-azidopyrene-labeled cytosol proteins. An equilibrium dissociation constant of 2.69 ± 0.66 nM and a maximum number of binding sites of 2.07 ± 0.10 nmol of [^{3}H]B[*a*]P bound/mg of protein were estimated for the pure protein. Two-dimensional gel electrophoresis further resolved the purified 29 000-dalton protein into three major isoelectric variants, each of which was specifically labeled by [^{3}H]-1-azidopyrene.

Polycyclic aromatic hydrocarbons are considered to be relatively inert molecules biologically until their metabolic transformation to reactive electrophilic species. The primary enzymes responsible for these reactions are the cytochrome P-450 system. Carcinogenesis by polycyclic hydrocarbons has been attributed to the covalent modification of cellular macromolecules by these chemically unstable hydrocarbon metabolites (Conney, 1982). The formation of DNA adducts in particular has been considered to be a critical initiating event in this process (Brookes & Lawley, 1964; Singer, 1964).

Recent investigations have demonstrated the existence of novel proteins that bind unmetabolized polycyclic aromatic hydrocarbons, and some polychlorinated compounds, in a reversible, noncovalent manner (Poland et al., 1976; Okey et al., 1979; Tierney et al., 1980; Zytkovicz, 1982; Collins & Marletta, 1984), and there has been increasing interest in the role these species may play in carcinogenesis. Binding affinities in the low nanomolar range and a finite number of sites per cell are the primary characteristics of these xenobiotic binding proteins. These features are reminiscent of hormone and drug receptor interactions (Burt, 1978). The TCDD[1] binding protein, or Ah receptor, from the C57B1/6J mouse is probably the best understood of these receptor-like proteins. Two distinct biological responses have been attributed to the Ah receptor. Its presence has been linked to the toxic manifestations of TCDD and chlorinated pesticide exposure in target tissues (Knutson & Poland, 1980, 1982), but the exact role of the receptor in this process is not yet understood. A second, better characterized function of the Ah receptor is the stimulation of microsomal drug-metabolizing activities. Independent investigations have shown that the Ah receptor participates in a mechanism of action analogous to steroid hormone receptors. Following binding of a number of polycyclic aromatic and chlorinated hydrocarbons to the receptor, stimulation of new cytochrome P-450 mRNA and protein synthesis is accomplished by DNA binding and enhancement of the transcription of cytochrome P-450 genes (Greenlee & Poland, 1979; Tukey et al., 1982; Jones et al., 1985).

Biochemical studies of the Ah receptor have identified a 9S sedimenting species in sucrose density gradients as a key component of the receptor (Okey et al., 1979; Hannah et al., 1981). Additional, more abundant binding species for polycyclic aromatic hydrocarbons have also been identified that are distinct from the Ah receptor. We (Collins & Marletta, 1984) and others (Holder et al., 1981; Zytkovicz, 1982) have described the ligand binding properties of these sites and some of their physical characteristics. It has been difficult to establish the biological significance of these binding sites, and it is tempting to speculate that some of these proteins may participate in the regulation of xenobiotic metabolism. A receptor role, similar to the Ah receptor, is one possibility. The existence of carrier proteins that may facilitate the metabolism of these lipophilic substrates has also been suggested, but supporting evidence remains inconclusive (Hanson-Painton et al., 1983).

Our studies have been directed toward the purification of these binding proteins. We considered this approach to be a valuable one for addressing the issue of their biological function. Isolation of the binding protein is a prerequisite for the preparation of antibodies that can be used as histochemical probes and as specific inhibitors in a test of biological function.

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[1] Abbreviations: Ah, aryl hydrocarbon; 1-AP–6B, 1-aminopyrene–Sepharose 6B; B[*a*]P, benzo[*a*]pyrene; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEDG buffer, 25 mM Hepes, 5.0 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol, pH 7.6; kDa, kilodalton; 3-MC, 3-methylcholanthrene; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TLC, thin-layer chromatography; UV, ultraviolet.

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The primary structure of the protein and the architecture of the binding site may also be studied in greater detail. Information obtained in our earlier ligand binding studies has allowed us to develop purification strategies that are based on the affinity of these proteins for aromatic hydrocarbon ligands. The affinity chromatography resin 1-aminopyrene–Sepharose 6B was developed for this purpose (Collins et al., 1985). In this paper we describe the preparation and use of a photoaffinity label for these proteins, and their purification is presented.

Materials and Methods

Animals. Male C57Bl/6J mice, age 7 weeks, were obtained from Jackson Laboratories (Bar Harbor, ME) and were housed at 22 °C, 50% relative humidity, in a fluorescent light controlled room. Charles River Mouse Chow and distilled water were given ad libitum for 1 week, and then mice were killed by cervical dislocation at age 8 weeks.

Chemicals. Reagent and specialty chemicals were obtained from the following sources: B[*a*]P, 1-aminopyrene, and 1-nitropyrene from Aldrich Chemical Co. (Milwaukee, WI) (*caution*: these compounds have been determined to be carcinogenic or cancer-suspect agents; handling of these compounds was carried out in a fume hood with protective clothing and gloves; solutions containing these compounds were combined and disposed of as biohazardous waste); palladium-coated polyethylenimine beads, epoxy-activated Sepharose 6B, and dextran from Sigma Chemical Co. (St. Louis, MO); DEAE-cellulose (DE52) from Whatman (Clifton, NJ); and Norit-A charcoal from Fisher Scientific (Pittsburgh, PA). Electrophoretic grade chemicals and SDS–polyacrylamide molecular weight standards (range 10 000–100 000) were obtained from Bio-Rad Laboratories (Richmond, CA).

[^{3}H]B[*a*]P (specific activity >78 Ci/mmol), ^{14}C-methylated ovalbumin (M_r 46 000), and ^{14}C-methylated BSA (M_r 69 000) were obtained from New England Nuclear (Boston, MA). [^{3}H]B[*a*]P was repurified in two steps as previously described (Collins & Marletta, 1984). [^{3}H]-1-Nitropyrene (23.7 Ci/mmol) was a generous gift from Dr. John Groopman, Boston University (Boston, MA).

Preparation of Liver Cytosol. Male C57Bl/6J mice were sacrificed at 8 weeks of age, and hepatic cytosol was prepared as previously described (Collins & Marletta, 1984). HDG buffer (HEDG buffer without EDTA) containing 0.5 mM PMSF was used as the homogenization buffer for cytosol that was applied to DEAE-cellulose. Protein concentrations were assayed by the method of Bradford (1976) or by the fluorescamine method (Udenfriend et al., 1972) with crystalline BSA as the standard.

Benzo[a]pyrene Binding Assay. Specific binding assays with [^{3}H]B[*a*]P were performed as previously described (Collins & Marletta, 1984), except that the volume of the dextran-coated charcoal suspension was 0.25 mL. In some experiments using partially purified proteins the sample volume of the assay was 0.25 mL, with all other component volumes reduced accordingly. For saturation binding studies with the purified protein the assay was modified to contain 1 mg/mL BSA and 2–5 μg/mL purified protein. This adjustment was necessary in order to maintain a protein concentration in the assay of 1.0 mg/mL. Binding incubations that contained 1-azidopyrene in either tritiated or nonradiolabeled form were performed at 10 °C for 2.5 h and were protected from light.

Electrophoresis and Isoelectric Focusing. SDS–polyacrylamide slab gel electrophoresis was carried out by using a modification of the method of Laemmli (1970) as previously described (Collins & Marletta, 1984). Apparent molecular weights were determined from a semilogarithmic plot of the molecular weight of the standards (Bio-Rad, Richmond, CA) phosphorylase *b* (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400) vs. migration distance. Gels were stained with silver (Morrissey, 1981). Isoelectric focusing was carried out as described in 3% polyacrylamide gels over a pH range of 4.5–7.1 (Tamkun & Hynes, 1983). Autoradiographs were prepared with Enlightening (New England Nuclear) and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY).

Synthesis of 1-Azidopyrene. The synthetic scheme for the preparation of 1-azidopyrene from 1-aminopyrene is essentially as described (Smith et al., 1981). Minor modifications of their procedure included doubling the molar equivalents of sodium nitrite and sodium azide, and the solvent system for silica gel chromatography was petroleum ether–methylene chloride (75:25). Purity was established by two-dimensional TLC (100% $CHCl_3$, first dimension; 100% petroleum ether, second dimension) and comparison to reported UV spectrum and mass spectrum.

Synthesis of [^{3}H]-1-Azidopyrene. To a 0.30-mL Reacti-vial (Wheaton, Millville, NJ) set in an 85 °C water bath was added 100 μCi (3.38 nmol) of [^{3}H]-1-nitropyrene dissolved in 200 μL of 95% (v/v) ethanol and 4 μL (82.3 nmol) of hydrazine hydrate. Addition of the catalyst (2.33 mg of palladium-coated polyethylenimine beads) was over a 3-h period. Progress of the reaction was monitored by TLC on 20-cm Bakerflex silica gel GF plates, which were developed in 100% CH_2Cl_2 alongside authentic standards of 1-nitro-, 1-amino-, and 1-azidopyrene. The plates were cut into 1-cm strips and analyzed for tritium by scintillation counting.

The reaction mixture from above was passed through a silanized Pasteur pipet containing crystals of $MgSO_4 \cdot 7H_2O$. The Reacti-vial and $MgSO_4$ were washed with a small volume of ethanol. The solvent was evaporated under a stream of nitrogen in a 0.30-mL Reacti-vial, and the [^{3}H]-1-aminopyrene was redissolved in 30 μL of acetone, 30 μL of H_2O, and 2.5 μL of 4 N HCl. The mixture was chilled on ice and sodium nitrite crystals were added in excess (0.78 mg, 14.7 μmol). The reaction was kept on ice for 1.5 h with brief mixing every 15 min, whereupon sodium azide (6.7 mg dissolved in 25 μL of H_2O) was added by micropipet. This reaction was monitored by TLC, and the product mixture was passed through a small amount of $MgSO_4$ as described above. The [^{3}H]-1-azidopyrene was purified by preparative silica gel TLC (1000-μm plates), eluted with CH_2Cl_2, and stored at −20 °C.

Photoaffinity Labeling by [^{3}H]-1-Azidopyrene and Analysis of Labeled Proteins. Sample proteins (0.1–2.5 mg/mL as indicated in figure legends) were incubated, protected from light, with 100–200 nM [^{3}H]-1-azidopyrene for 2.5 h on ice or were preincubated for 2 h with either 5 or 12.5 μM B[*a*]P, as indicated in figure legends, prior to the addition of [^{3}H]-1-azidopyrene for 5 min. After brief treatment with dextran-coated charcoal to remove unbound ligand, samples were irradiated for 15–20 min at 300 nm in a Rayonet photochemical reactor (Southern New England Ultraviolet Co., Hamden, CT). The quartz reaction vessel was maintained at 4 °C by an internal water jacket. Some samples, due to their small volume (<100 μL), were not pretreated with charcoal and were irradiated for 15 min in 6-mm diameter quartz tubes without cooling. Aliquots from each reaction were electrophoresed through a 1-mm-thick 12.5% SDS–polyacrylamide gel as described above. The gel was fixed, sliced, and dissolved in 1.0 mL of 30% H_2O_2 overnight at 50 °C and counted for

tritium. Some samples were subjected to isoelectric focusing prior to SDS–polyacrylamide gel electrophoresis or were fixed without staining and prepared for autoradiography.

Gel Filtration Chromatography. Sephacryl S-300 Superfine (Pharmacia, Piscataway, NJ) was equilibrated in HEDG buffer containing 0.1 M NaCl and 0.02% sodium azide at 4 °C (Poellinger et al., 1983) and poured into a 1.5-cm diameter column (bed height 110 cm). The flow rate was maintained at 4 mL/h with a peristaltic pump. The column was calibrated with the following standards applied in 0.5-mL volumes: Blue Dextran (2 mg/mL, 2000000 daltons), β-amylase (4 mg/mL, 200000 daltons), alcohol dehydrogenase (5 mg/mL, 150000 daltons), bovine serum albumin (10 mg/mL, 66000 daltons), carbonic anhydrase (3 mg/mL, 29000 daltons), and cytochrome *c* (2 mg/mL, 12400 daltons). Fractions (40 drops) were collected, and protein was monitored spectrophotometrically at 280 nm.

Purification of Carcinogen Binding Protein from Mouse Liver. (1) DEAE-cellulose Chromatography. DEAE-cellulose (Whatman DE52) was equilibrated in 200 mM Hepes, 1 mM DTT, and 10% glycerol, pH 7.50, at 4 °C with several changes, and the pH of the slurry was adjusted to 7.50 with HCl. The DE52 slurry was poured into a Pharmacia K50/30 column (5-cm diameter) at a flow rate of 1–1.5 mL/min. The packed column (bed volume 360 mL) was washed with HDG buffer until the pH and conductivity of the input and effluent buffers were identical (about 50 h).

From 50 C57B1/6J mice, liver cytosol (3.73 g of protein in 246 mL) was prepared in HDG buffer containing 0.5 mM PMSF. DEAE chromatography of this cytosol was carried out in two batches to avoid overloading of the column. One-third of the sample was frozen in 4-mL aliquots at −80 °C, and the remainder was pumped onto the column at 0.7 mL/min and fractions (8 mL) were collected. Protein concentration in selected fractions was monitored (Bradford, 1976). Protein that did not bind to the resin was eluted. When a steady base line was obtained, a linear gradient of NaCl from 0 to 500 mM in HDG buffer was applied (1.8-L total gradient volume) and eluted proteins in each fraction were monitored. Upon completion of the gradient the NaCl concentration was raised to 1 M in HDG as the final column-washing step. Proteins eluted during the salt gradient were pooled, concentrated on an Amicon YM5 membrane under 40 psi N_2, and dialyzed against HEDG containing 0.1 M NaCl. Aliquots of the concentrated fractions were adjusted to 1.0 mg/mL and assayed for specific [^{3}H]B[*a*]P binding activity. The DEAE resin was recycled by washing with 2 M NaCl and 2 M urea in 0.2 M Hepes, pH 7.5 (1 L). Following reequilibration of the column with HDG buffer the remaining cytosol was thawed and chromatographed.

(2) Affinity Chromatography. 1-Aminopyrene–Sepharose 6B (1-AP–6B) (1.0 g desiccated weight) was equilibrated in HEDG buffer containing 0.1 M NaCl, pH 7.5, as previously described (Collins et al., 1985). The beads were collected on a glass frit and washed with equilibration buffer. Protein containing B[*a*]P binding activity from DEAE-cellulose chromatography was concentrated to a volume of 250 mL and mixed with the equilibrated 1-AP–6B in a 250-mL Nalgene centrifuge bottle. The suspension was mixed at a moderate speed on an end-over-end shaker apparatus for 24 h at 4 °C. The suspension was poured into a 1.5 × 10 cm column, and the resin was packed at a flow rate of 0.16 mL/min (bed height 5.5 cm). The proteins were cycled over the packed bed for 20–24 h, and then unbound proteins from the column were collected. The 1-AP–6B column was washed with equilibration buffer (approximately 200 mL) to remove any nonspecifically bound material. Elution of protein bound to 1-AP–6B was performed with a gradient (22 mL) from 0 to 2.0 M NaSCN in HEDG buffer, pH 7.50 (flow rate of 0.14 mL/min, 5 min/fraction), followed by an additional 25 mL of the limit buffer. Most of the protein eluted in a broad peak between fractions 14 and 50, as monitored by absorbance at 280 nm with correction for absorbance due to NaSCN in buffer alone. The fractions within this region were pooled and dialyzed against HEDG buffer to remove the thiocyanate. A final concentration of the sample on an Amicon YM5 membrane yielded 14.5 mL with a protein concentration of 0.125 mg/mL as measured by fluorescamine assay (Udenfriend et al., 1972).

Curve Fitting and Data Analysis. Equilibrium dissociation constants and maximum number of binding sites were calculated, with their standard errors, from saturation ligand binding data by a least-squares curve-fitting procedure (Barlow, 1984). This method also provided coefficients of multiple correlation and the standard error of the fit to a single-site binding model.

Competition binding data (expressed as the fraction of control binding vs. the logarithm of unlabeled competing ligand) were analyzed by nonlinear regression. The iterative curve-fitting routine estimated the number of binding sites, the proportion of specific binding attributed to each site, and their IC_{50} values (courtesy of M. B. Bolger, University of Southern California).

RESULTS

*Inhibition of [^{3}H]B[*a*]P Binding by 1-Azidopyrene.* Data from competition binding studies between [^{3}H]B[*a*]P and 1-azidopyrene were fit by the iterative procedure described under Materials and Methods. The top panel of Figure 1 indicates that 1-azidopyrene inhibits specific [^{3}H]B[*a*]P binding. At a 1-azidopyrene concentration of 57.7 nM control binding was inhibited by 50%. Further analysis of these data indicated a best fit to a single class of binding sites. The data could not be fit to a two-site model. When multiple inhibition curves were generated and the data analyzed by a double-reciprocal plot (Lineweaver & Burk, 1934) (Figure 1, bottom), a model of competitive inhibition was supported, with a calculated K_i for 1-azidopyrene of 35.0 ± 5.7 nM.

Affinity Labeling with [^{3}H]-1-Azidopyrene in 100000g Mouse Hepatic Cytosol. Hepatic cytosol proteins were photoaffinity labeled with [^{3}H]-1-azidopyrene in the absence or presence of unlabeled B[*a*]P and were separated electrophoretically through a 12.5% SDS–polyacrylamide gel to examine the labeling pattern. Slices of the gel (5 mm) were counted for tritium incorporated into protein. Figure 2 illustrates the result. The level of spurious background labeling throughout the gel was low. Unincorporated label migrated with the dye front. A peak of apparent molecular weight 29000 was observed, whose formation was blocked by preincubation with 5 μM unlabeled B[*a*]P, as would be expected from the competitive inhibition data (Figure 1). A much smaller and more diffuse species of approximately 50000 daltons was also observed, but was absent from all subsequent affinity labeling results. When portions of these samples were further analyzed by sucrose density gradient centrifugation, a single peak with a sedimentation value of 4 S was observed (not shown).

Molecular Weight Determination of the Binding Protein by Gel Filtration Chromatography. C57B1/6J mouse liver cytosol that had been preincubated with either [^{3}H]B[*a*]P or [^{3}H]-1-azidopyrene possessed similar relative mobilities of 33 ± 5 ([^{3}H]B[*a*]P) and 29 ± 5 ([^{3}H]-1-azidopyrene) kDa on a column of Sephacryl S-300 Superfine. The calibration curve (not shown) was generated as described under Materials and

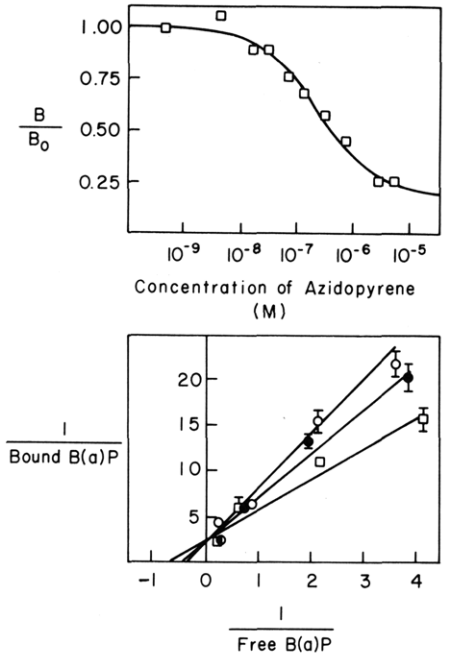

FIGURE 1: 1-Azidopyrene competition and competitive inhibition. (Top) 1-Azidopyrene IC_{50}. B6 mouse hepatic cytosol (1.0 mg/mL in HEDG buffer) was incubated with [^{3}H]B[*a*]P (2.1 nM) plus increasing concentrations of 1-azidopyrene for 75 min at 10 °C protected from light. Specific binding was measured by the dextran-coated charcoal assay. Data were fitted by a least-squares nonlinear curve-fitting routine as described under Materials and Methods. IC_{50} = 57.7 nM; fraction of nonspecific binding = 0.18. (Bottom) Double-reciprocal analysis of inhibition of [^{3}H]B[*a*]P binding by 1-azidopyrene. Sample incubation conditions and measurement of specific binding were determined as above. Concentrations of [^{3}H]B[*a*]P were between 0.30 and 5.0 nM in the presence of 0 (□), 20 (●), or 50 (○) nM 1-azidopyrene. Nonspecific binding was measured in the presence of 1 μM B[*a*]P.

Methods. From these data the molecular weight of the protein by SDS gel electrophoresis appears to be the native molecular weight.

Chromatography of B6 Hepatic Cytosol on DEAE-cellulose. The protein profile from anion-exchange chromatography is depicted in Figure 3. The recovery of total protein from the column was 95%. Subsequent dialysis and pressure concentration steps resulted in some loss due to precipitation and membrane binding. Points of fractionation were based on preliminary experiments that monitored the elution of preformed [^{3}H]B[*a*]P–protein complexes. Aliquots from the regions designated I–VI are displayed in the silver-stained gel of Figure 4. Almost half of the total protein applied to the column was not retained and eluted as a sharp peak (I; lane 2 of Figure 4). It was similar in composition to the unfractionated cytosol starting material (lane 1 of Figure 4). A second small peak (II; lane 3 of Figure 4) eluted before initiation of the salt gradient. Spectral characteristics indicated that this material was oxyhemoglobin (Waterman, 1978).

Regions I–VI from the column were examined for specific [^{3}H]B[*a*]P binding activity by a single-point (20 nM [^{3}H]B[*a*]P ± 1 μM B[*a*]P) binding assay. The results, as shown in Table I, indicated that region IV contained elevated levels of specific [^{3}H]B[*a*]P binding. While other protein fractions from the column, in particular regions V and VI, exhibited comparatively high levels of binding, it appeared to be nonspecific. This anion-exchange step achieved an estimated 2.5-fold purification of specific [^{3}H]B[*a*]P binding activity over the 100000*g* supernatant starting material.

Affinity Chromatography on 1-Aminopyrene–Sepharose 6B. From 320 ± 5.7 mg (mean ± SD of duplicate determinations) of total protein in DE52 fraction number IV, 310 ± 3.7 mg was collected from the aminopyrene–Sepharose 6B column as the unbound effluent (not shown).

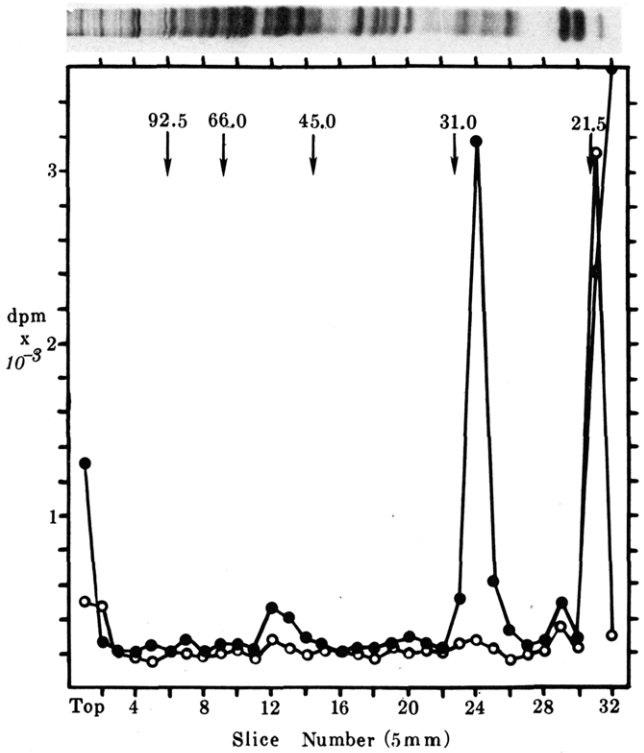

FIGURE 2: Analysis of [^{3}H]-1-azidopyrene photoaffinity labeled polypeptides from hepatic cytosol separated by SDS–polyacrylamide gel electrophoresis. Sample proteins (2.5 mg/mL in HEDG buffer) were incubated, protected from light, with 150 nM [^{3}H]-1-azidopyrene for 2 h on ice [total labeling (●)] or were preincubated for 2 h with 5 μM B[*a*]P prior to the addition of 150 nM [^{3}H]-1-azidopyrene for 5 min [site-protected labeling (○)]. Preparation and irradiation of the samples were performed as described under Materials and Methods. Aliquots (75 μg) from each reaction were electrophoresed through a 12.5% polyacrylamide gel. The gel was fixed, sliced, and dissolved in 1.0 mL of 30% H_2O_2 overnight at 50 °C and counted for tritium. Migration of molecular weight marker proteins (in kilodaltons) is indicated. At the top of the figure cytosolic proteins, separated by SDS–PAGE and stained with silver, are displayed in the same orientation as the figure.

Table I: [^{3}H]B[*a*]P Binding Activity[a] in DEAE-cellulose Column Fractions[b]

sample	total	nonspecific	specific
cytosol	2.805	1.302	1.503
DE52-I	0.961	0.680	0.281
DE52-II	nd[c]	nd	
DE52-III	1.951	2.249	0
DE52-IV	5.865	3.104	2.761
DE52-V	3.900	3.151	0.751
DE52-VI	3.173	3.405	0

[a] Picomoles per milligram of protein. [b] Samples were adjusted to 1.0 mg/mL protein with HEDG containing 0.1 M NaCl after concentration on an Amicon YM5 membrane. The concentration of [^{3}H]B[*a*]P was 20–25 nM. Nonspecific binding was measured in the presence of 1 μM B[*a*]P. [c] Not determined; in previous experiments this fraction displayed no binding.

The final product isolated by affinity chromatography (1.81 mg of protein) was electrophoresed through a 12.5% SDS–polyacrylamide gel and stained with silver (Figure 5, left). A single protein band of 29.5 kDa was found, with a minor contaminant apparent at approximately 32 kDa. The pair of more faintly staining bands in the 60-kDa range are staining artifacts derived from the β-mercaptoethanol in the sample buffer (Merril et al., 1984). Photoaffinity labeling with

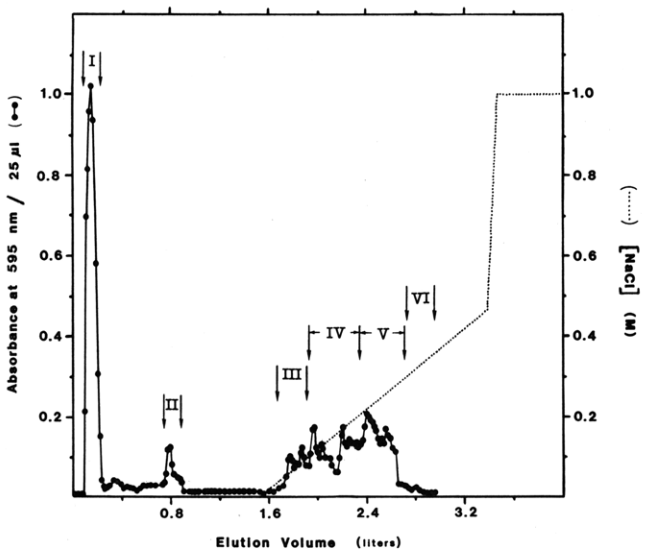

FIGURE 3: DEAE-cellulose chromatography of hepatic cytosol. Mouse liver cytosol (2.64 g in 162 mL) was applied to a 5.0 × 18.5 cm column of Whatman DE52, which was equilibrated in HDG buffer (pH 7.5) at a flow rate of 0.75 mL/min. Fractions (8.0 mL) were collected and monitored for protein (●). When a steady base line was obtained, a linear gradient (···) from 0 to 500 mM NaCl was applied (1.8 L total gradient volume) and proteins bound to the resin were collected in fractions. The regions indicated (I–VI) were pooled, concentrated on an Amicon YM5 membrane, dialyzed against HEDG buffer containing 0.1 M NaCl, and assayed for specific [^{3}H]B[*a*]P binding activity.

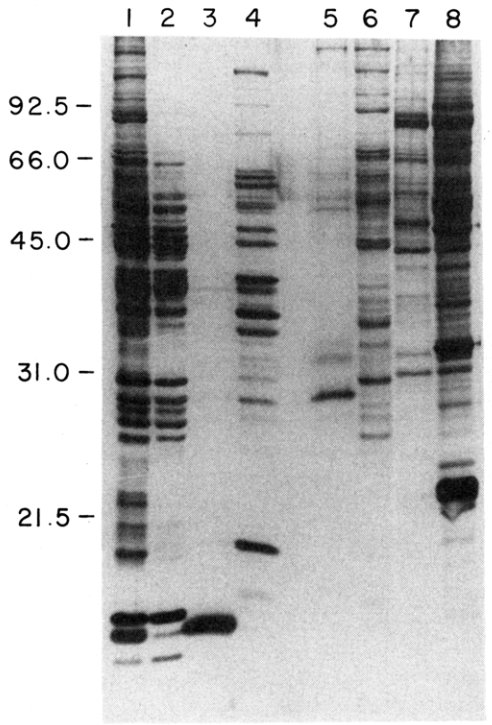

FIGURE 4: SDS–PAGE of DEAE-cellulose column fractions and purified protein after affinity chromatography. Lanes: (1) whole cytosol (16 µg); (2) DE52 fraction I (8.2 µg); (3) DE52 fraction II (5.5 µg); (4) DE42 fraction III (8.8 µg); (5) protein purified by affinity chromatography (5.5 µg); (6) DE52 fraction IV (10 µg); (7) DE52 fraction V (9.1 µg); (8) DE52 fraction VI (16.4 µg). Electrophoresis was through a 12.5% polyacrylamide gel stained with silver. Mobility of molecular weight standards (in kilodaltons) indicated along margin.

[^{3}H]-1-azidopyrene (Figure 5, right) confirmed this 29-kDa species as the carcinogen binding protein, in agreement with and as expected from the affinity labeling results from unfractionated cytosol (Figure 2). The binding of [^{3}H]-1-azidopyrene by the protein (Figure 5, right, lane 1) and the absence of labeling in the presence of excess unlabeled B[*a*]P (Figure 5, right, lane 2) indicated the retention of specific binding activity.

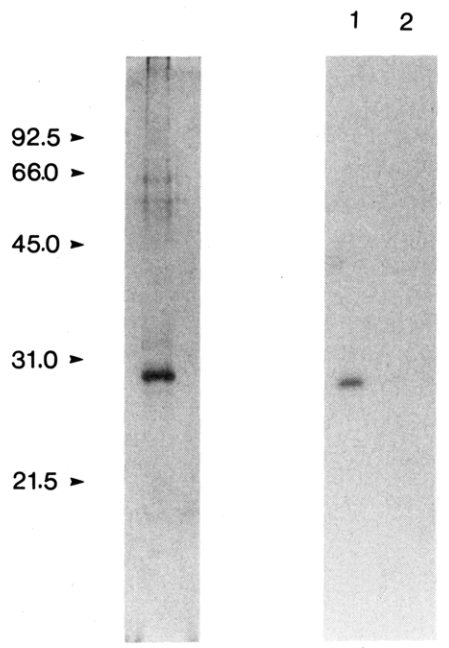

FIGURE 5: SDS–polyacrylamide gel electrophoresis of purified binding protein. Electrophoresis (7.2 µg) was through a 12.5% polyacrylamide gel: left, silver staining; right, radioautography of binding protein that was affinity labeled with [^{3}H]-1-azidopyrene prior to electrophoresis. [^{3}H]-1-Azidopyrene (approximately 75 nM) was incubated in the absence (lane 1) or presence (lane 2) of 12.5 µM unlabeled B[*a*]P. Molecular weight standards (in kilodaltons) indicated.

The purification of the hepatic B[*a*]P binding protein is summarized in Table II. The binding parameters were determined at each step by saturation ligand binding experiments (Collins & Marletta, 1984). The dissociation constants obtained (expressed as the mean ± SE of the fit) were 3.15 ± 1.7 nM (cytosol), 4.02 ± 0.33 nM (DE52 fraction IV), and 2.69 ± 0.66 nM (purified). These values were in reasonable agreement with each other and indicated that purification of the protein did not result in significant changes in binding affinity. For each of these three samples, the data were also consistent with a single-site binding model. The estimations for B_{max} in cytosol and in DE52 fraction IV were 5.48 ± 0.77 and 13.56 ± 0.46 pmol of [^{3}H]B[*a*]P bound/mg of protein, respectively. Even though the corresponding values presented in Table I for specific [^{3}H]B[*a*]P binding in cytosol and DE52 fraction IV were 4-fold lower, from both sets of data a purification factor of 2–2.5 was estimated. These differences probably reflect the fact that saturation binding studies measure total number of binding sites present. For this reason binding parameters derived from curve fitting are more reliable than single-point or other nonequilibrium methods, such as sucrose density gradients. The maximum number of binding sites for the purified protein was 2070.0 ± 104.0 pmol of [^{3}H]B[*a*]P bound/mg of protein. From these specific activity measurements, affinity chromatography produced a 150-fold purification, from which a final purification factor of 380 was calculated. Other evidence, however, indicates a much higher level of purity. Scanning densitometry of the protein profiles from Figure 4 before (lane 6) and after (lane 5) aminopyrene–Sepharose affinity chromatography estimated the purification of the 29-kDa protein at this step to be at least 620-fold, which would raise the final purification factor above 1500.

Table II: Purification Table

step	protein (mg)	total activity (pmol)	yield (%) step	yield (%) total	specific activity[a,b]	x-fold purification[c] step	x-fold purification[c] total
cytosol	2643	14483.6	100	100	5.48 ± 0.77	1	1
DEAE-cellulose	320	4339.2	29.8	29.8	13.56 ● 0.46	2.5	2.5
aminopyrene–Sepharose	1.81[d]	3752.6	86.5	25.8	2070.4 ± 104.0	152.7	381.7

[a] Picomoles of [^{3}H]B[*a*]P bound per milligram of protein. [b] Values determined by saturation binding and estimated by least-squares curve fitting. [c] The theoretical purification for 1:1 binding stoichiometry of a 30 000-dalton protein is 6050-fold, which equals 33.3 nmol/mg theoretical specific activity divided by 5.5 pmol/mg observed cytosol specific activity. [d] Protein concentrations at this step determined by fluorescamine assay.

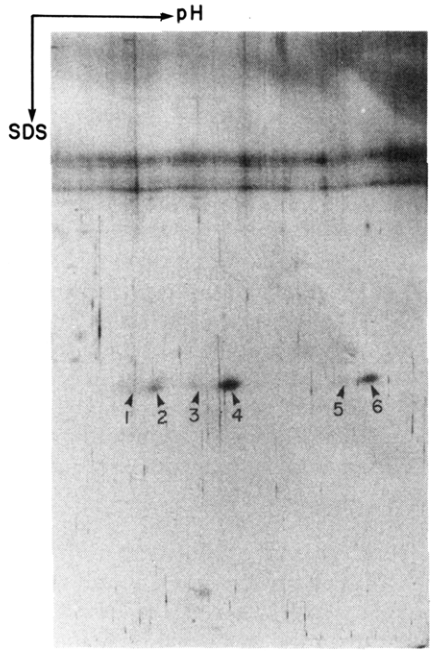

FIGURE 6: Two-dimensional gel analysis of purified carcinogen binding protein. The purified protein (12.5 μg in 100 μL of HEDG buffer) was photoaffinity labeled with [^{3}H]-1-azidopyrene by procedures described under Materials and Methods. Electrophoresis in the isoelectric focusing dimension was from right (basic) to left (acidic) and in the SDS dimension from top to bottom (12.5% separating gel, 3% stacking gel). Total protein applied was 5 μg. Proteins were visualized by silver staining. Spots numbered 1–6 (from left to right) at 29 kDa were punched out of the gel with a cork borer, dissolved in 1.0 mL of 30% H_2O_2 overnight, and counted by liquid scintillation counting for incorporation of tritium from [^{3}H]-1-azidopyrene.

Two-Dimensional Gel Analysis of Purified Carcinogen Binding Protein. The protein purified by affinity chromatography on aminopyrene–Sepharose was subjected to two-dimensional gel electrophoresis following affinity labeling with [^{3}H]-1-azidopyrene. The silver-stained gel is presented in Figure 6. Isoelectric focusing resolved the 29-kDa protein into three major, and possibly three minor, components. A small cluster of faintly staining spots was observed in the acidic portion of the gel in the 30–40-kDa range, indicating the presence of some minor contaminants. Control samples that had not been affinity labeled prior to the focusing procedure generated an identical staining pattern (not shown), eliminating the possibility that isoelectric variants at 29 kDa were generated by the affinity labeling procedure itself.

The isoelectrically distinct 29-kDa polypeptides were excised from triplicate two-dimensional gels and examined for incorporation of [^{3}H]-1-azidopyrene. Figure 7 depicts the results as a histogram. Numbers below each bar refer to individual spots as indicated in Figure 6. The starred (*, **) bars are background measurements as described in the legend to Figure 7. All three major (numbered 2, 4, 6) and the three minor (numbered 1, 3, 5) 29-kDa species show incorporation of label from [^{3}H]-1-azidopyrene above background levels. The amount of label appears to be in proportion to the amount of protein in each spot.

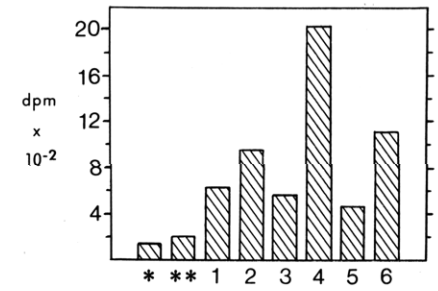

FIGURE 7: Incorporation of [^{3}H]-1-azidopyrene into isoelectric variants at 29 kDa. Spots from two-dimensional gels as indicated in Figure 7 were excised from triplicate gels and counted for incorporated tritium as described under Materials and Methods. Data are expressed as dpm in each spot. Background in gel where no protein present (*); background labeling in a region of gel containing minor contaminant proteins (**).

Discussion

Three major isoelectric variants of the B[*a*]P binding protein from mouse liver were isolated by our purification scheme. The synthesis and development of an affinity chromatography method and a specific photoaffinity label allowed us to purify this protein in two simple chromatographic steps. We have recently described the affinity chromatography resin, 1-aminopyrene–Sepharose 6B, as a selective tool for the purification of binding proteins of this type (Collins et al., 1985). In this paper we have demonstrated the use of 1-azidopyrene as a specific affinity labeling agent for the B[*a*]P binding protein from mouse liver. Our ability to label this protein with [^{3}H]-1-azidopyrene in whole cytosol and in purified preparations was an integral component of this purification strategy. Using this method, we were also able to show that the multiple 29-kDa species separated by two-dimensional gel electrophoresis were each capable of binding ligand and represent isoelectric variants.

From our purification scheme a single 29-kDa protein was isolated (Figure 5). Specific binding parameters were estimated at each step of the purification by saturation binding analyses. Binding affinity did not significantly change over the course of purification, and the specific activity of the purified protein was 2.07 ± 0.10 nmol of [^{3}H]B[*a*]P bound/mg of protein.

From the partial purification of a related 3-MC binding protein from rat liver cytosol (Tierney et al., 1983), a specific activity of 0.10 nmol of [^{3}H]-3-MC bound/mg of protein was reported. An abundant 32-kDa species in their preparation, visualized by SDS–PAGE and silver staining, may be analogous to the 29-kDa protein from mouse liver. However, other molecular weight species of 40, 25, and 14 kDa were also reported by these investigators to have carcinogen binding activity, which contrasts with the single 29-kDa protein that we have isolated. Since these additional molecular weight

species were not purified to homogeneity, one cannot rule out the possibility that they were contaminants of the 32-kDa activity. This situation would also contribute to a lower specific activity.

One question that arose during the initial affinity labeling studies in whole cytosol was whether the binding protein was composed of one or more polypeptides under native conditions. Gel filtration chromatography, under nondenaturing conditions, was used to address this concern. The values obtained with [^{3}H]B[*a*]P- and [^{3}H]-1-azidopyrene-labeled cytosolic proteins were in reasonable agreement with each other and with the size estimated by SDS gel electrophoresis. These results indicated that under both native and denaturing conditions the binding protein is composed of a single polypeptide of approximately 30 kDa. However, the possibility that this protein is derived from a larger polypeptide that has been cleaved by proteolysis during or following tissue homogenization cannot be excluded. Our findings differ from those obtained in other studies of polycyclic hydrocarbon binding proteins. Other investigators have reported considerably higher values for B[*a*]P and 3-MC binding proteins estimated by gel filtration of rat and mouse cytosol. An apparent molecular weight of 61 000 was reported (Zytkovicz, 1982) for a B[*a*]P binding protein in cultured mouse embryo cells, while the molecular weight of a hepatic 3-methylcholanthrene binding protein has been variously placed at 45 000 (Tierney et al., 1980), 49 000 (Tierney et al., 1983), and 87 000 (Hannah et al., 1981). The basis for this variation is unknown. Some have suggested a subunit structure for these proteins composed of lower molecular weight polypeptides (Tierney et al., 1983). We find no evidence for such a subunit model. While some data indicate that these proteins are very similar, their identity has not yet been rigorously established. As these other binding proteins are purified, direct comparisons between them can be made. The procedure that we describe here should be useful for this purpose.

From an analysis of the purification table (Table II), based on ligand binding data, a final purification factor of 380 was estimated for the mouse liver B[*a*]P binding protein. This value would represent 6% purity based on a theoretical enrichment factor of 6000, assuming a 1:1 binding stoichiometry. However, by SDS gel electrophoresis and affinity labeling it would appear that the protein has been purified to homogeneity. In addition, scanning densitometry of samples before and after affinity chromatography raises the estimate of purification at this step alone to at least 600. Taken together, these observations suggest that the *x*-fold purification derived from specific activity considerations is underestimated.

The recovery of protein from our purification was also greater than expected from the initial activity present in cytosol, again based on a 1:1 ratio of ligand–protein. One factor that may contribute to an excessive yield of protein with a less than theoretical specific activity is the presence of a major contaminant in the final preparation. While isoelectric focusing revealed multiple 29-kDa spots (Figure 6), affinity labeling of each of these ruled out the presence of a contaminant as a source of this inconsistency (Figure 7). For a number of hormone and drug receptors that have been purified, similar discrepancies between theoretical and actual purification have been noted.

The following observations from the literature serve to illustrate this point. Receptors for insulin (Jacobs et al., 1977; Siegel et al., 1981), prolactin (Liscia & Vonderhaar, 1982), steroid hormones (Coty et al., 1979; Kuhn et al., 1975), vitamin D (Simpson & DeLuca, 1982; Simpson et al., 1983), acetylcholine (Lindstrom et al., 1983), and the GABA/benzodiazepine complex (Sigel et al., 1983) were purified to apparent homogeneity but were accompanied, in each case, by a less than theoretical recovery of ligand binding activity. The two most frequently identified sources of discrepancy were inactivation of the receptors during purification and underestimation of the initial levels of activity present in the starting material. In the first case, high salt concentrations, extremes of pH, and absence of ligand or other stabilizing factors contributed to the inactivation of a proportion of the receptors. In many instances, modifications of the original procedures have led to the isolation of fully or nearly fully active receptors (Sica et al., 1972; Fujita-Yamaguchi et al., 1983; Sigel & Barnard, 1984; Necessary et al., 1984). The lowered specific activity of the mouse liver carcinogen binding protein that we have purified may be due, in part, to a similar process of protein inactivation. Note, however, that binding activity must have been retained at least through the affinity chromatography step.

In the second case, underestimates of specific binding activity in tissue homogenates or subcellular fractions with yields of greater than 100% have, in some instances, been shown to result from the separation of inhibitors or endogenous ligands during the course of purification. In several hormone receptor systems, the presence of endogenous hormone at the start of the purification has greatly underestimated the total number of binding sites present and consequently inflated the degree of purification required (Schrader et al., 1977; Schrader & O'Malley, 1978; Coty et al., 1979; Greene et al., 1981; Simpson & DeLuca, 1982; Simpson et al., 1983; Kelly et al., 1979). The existence of endogenous ligands or inhibitors for the mouse liver carcinogen binding protein has not been extensively investigated and could contribute to underestimates of the number of cytosolic binding sites. In preliminary experiments, we searched for low molecular weight inhibitors in mouse serum, but none have been identified. Further investigation of this question is in progress.

The isolation of this polycyclic hydrocarbon binding protein from mouse liver is a key step toward understanding both the biophysical factors involved in the binding of B[*a*]P and other hydrophobic substances and the biochemical functions of this interesting class of proteins. Their role as receptors or carrier proteins, two activities that have been proposed (Hanson-Painton et al., 1983; Tierney et al., 1983), can be further investigated. Our identification of charge variants of the 29-kDa binding protein was an unexpected and intriguing finding. These isoelectric variants may represent different posttranslational products. Phosphorylation is one such modification that has been frequently observed to participate in the modulation of enzyme activities and receptor–effector coupling. The preparation of antibodies directed against this binding protein should be extremely valuable in this regard. Tissue distribution studies by ligand binding indicated that binding activity is most abundant in liver and low to nonexistent in other tissues (Collins, 1985). These findings can be extended to the subcellular localization of the protein by an immunohistochemical approach, which minimizes the disruption of cellular architecture. The availability of pure protein now makes the study of many of these issues possible.

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Registry No. B[*a*]P, 50-32-8; 1-azidopyrene, 36171-39-8; 1-aminopyrene, 1606-67-3; [^{3}H]-1-nitropyrene, 102920-90-1; [^{3}H]-1-aminopyrene, 102870-97-3; [^{3}H]-1-azidopyrene, 102870-98-4.

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