Implication of the "4S" Polycyclic Aromatic Hydrocarbon Binding Protein in the Transregulation of Rat Cytochrome P-450c Expression†

William H. Houser,‡ Ronald N. Hines,‡,§ and Edward Bresnick*,‡,§,∥

Eppley Institute for Research in Cancer and Allied Diseases and Departments of Biochemistry and Pharmacology, The University of Nebraska Medical Center, Omaha, Nebraska 68105

Received April 25, 1985

ABSTRACT: A protein which specifically binds [^{3}H]benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons has been purified over 6000-fold from rat hepatic cytosol by using ion-exchange, gel permeation, and hydrophobic interaction chromatography. The binding protein differs from the 9S binding protein characterized in other laboratories. A Stokes radius of 2.75 nm was determined by gel filtration on Sephadex G-100. A sedimentation coefficient of 3.3 S was determined by using sucrose gradient analysis. The ability of this protein to bind total rat liver DNA as well as subclones containing portions of the rat cytochrome P-450c gene was investigated. Under high stringency conditions, this binding protein was found to interact in a specific and saturable manner with several subclones of the rat cytochrome P-450c gene containing 5′-upstream sequences, as well as portions of intron 1. Binding was not observed to the coding portions of the gene. These data implicate the "4S" binding protein in the transregulation of rat cytochrome P-450c expression.

The biotransformation of foreign compounds to toxic metabolites has been implicated in a variety of disorders ranging from tissue necrosis to mutagenesis and carcinogenesis (Brodie et al., 1971; Weisburger & Weisburger, 1973; Miller & Miller, 1966). The cytochromes P-450 are a family of monooxygenases that participates in the metabolic activation of a variety of substrates (Conney, 1967; Gillette et al., 1972). Rat cytochrome P-450c, the isozyme most closely associated with aryl hydrocarbon hydroxylase (AHH)[1] activity, is responsible for the metabolism of many PAHs such as benzo[*a*]pyrene (BP) and 3-methylcholanthrene (3MC) (Ryan et al., 1979).[2] The mechanisms whereby the expression of AHH activity is regulated have been investigated for many years (Bresnick et al., 1967; Guenthner & Nebert, 1977; Poland et al., 1976; Okey et al., 1979; Tierney et al., 1980). It is now known that treatment of animals with any of a variety of PAHs results in the coordinate induction of several drug-metabolizing enzymes collectively called the Ah locus. The regulation of induction of these enzymes has been studied most extensively in the mouse (Nebert et al., 1984; Kimura et al., 1984). The genetic responsiveness to PAH induction of AHH activity has been correlated strongly with the presence of a cytosolic binding protein (Guenthner & Nebert, 1977; Okey et al., 1979). This binding protein has been shown to be saturable and to bind TCDD and PAH reversibly with high affinity. Interestingly, analysis of the binding proteins by sucrose density gradients, gel filtration, and substrate specificity indicates at least two distinct high-affinity binding proteins which might play a role in the regulation of expression of AHH activity. Several investigators have reported a 4–4.5S protein that displays saturable, high-affinity binding to substrates such as 3MC and BP (Tierney et al., 1980, 1983; Heintz et al., 1981; Zytkovicz, 1982; Collins & Marletta, 1984). Other investigators (Okey et al., 1979, 1984; Nebert et al., 1984) have described an 8–9S protein that displays saturable, high-affinity binding to TCDD and a variety of PAHs.

The purpose of this study was to investigate the potential role of the "4S" binding protein in the regulation of expression of cytochrome P-450c in the rat. We herein describe a simplified purification of the "4S" binding protein. In addition, we have investigated the interaction of this binding protein under high stringency conditions with total rat liver DNA and with three subclones of the cytochrome P-450c gene.

Materials and Methods

Chemicals. Generally ^{3}H-labeled benzo[*a*]pyrene (specific activity 70 Ci/mmol) and deoxycytidine 5′-α-[^{35}S]thiotriphosphate (1300 Ci/mmol) were obtained from Amersham, Arlington Heights, IL. Sephacryl S-200, DEAE-Sepharose, Sepharose-CM, Sephadex G-100, Sephadex G-50, phenyl-Sepharose, and calibration proteins for column chromatography were obtained from Pharmacia, Inc., Piscataway, NJ. Benzo[*a*]pyrene, dithiothreitol, phenylmethanesulfonyl fluoride, streptomycin sulfate, Na_2EDTA, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, lauryl sulfate, glycine, *N*,*N*,*N*′,*N*′-tetramethylenediamine, and Trizma Base

†This work was supported by National Institutes of Health Grant CA36106-02 and by Laboratory Cancer Research Core Grant CA36727 from the National Cancer Institute.

* Address correspondence to this author at the Eppley Institute for Research in Cancer and Allied Diseases.

‡ Eppley Institute for Research in Cancer and Allied Diseases.

§ Department of Biochemistry.

∥ Department of Pharmacology.

[1] Abbreviations: 3MC, 3-methylcholanthrene; BP, benzo[*a*]pyrene; [^{3}H]BP, generally labeled benzo[*a*]pyrene; TCDD, tetrachlorodibenzodioxin; PAH, polycyclic aromatic hydrocarbon; HAP, hydroxylapatite; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DBA, dibenz[*a*,*h*]anthracene; A_{280}, absorbance at 280 nm; AHH, aryl hydrocarbon hydroxylase; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; kb, kilobase(s); bp, base pair(s); kbp, kilobase pair(s); BSA, bovine serum albumin.

[2] For this study, we have adopted the nomenclature proposed by Levin and Thomas (Ryan et al., 1979). The major cytochrome P-450 isozyme induced by 3MC in the rat is designated as cytochrome P-450c. Cytochrome P-450c in the rat corresponds to mouse cytochrome P1-450 and rabbit cytochrome P-450 form 6. Rat cytochrome P-450c, mouse cytochrome P1-450, and rabbit cytochrome P-450 form 6 are associated primarily with aryl hydrocarbon hydroxylase activity.

0006-2960/85/0424-7839$01.50/0

were obtained from Sigma Chemical Co., St. Louis, MO. Tween 80 was obtained from Fisher Scientific Co., St. Louis, MO. Affi-Gel Blue, acrylamide, *N,N'*-methylenebis(acrylamide), Bio-Rad silver strain, and hydroxyapatite (HAP) were obtained from Bio-Rad, Richmond, CA. *Hin*dIII restriction endonuclease was obtained from New England Biolabs, Beverly, MA. T4 DNA polymerase was obtained from Bethesda Research Laboratories, Inc., Gaithersburg, MD. Nitrocellulose filters (25 mm, 0.45 μm) were purchased from Gelman Sciences, Ann Arbor, MI.

Preparation of Cytosolic Fraction. Male Sprague-Dawley rats (150–250 g) were obtained from Sasco, Inc., Omaha, NE. Animals were sacrificed by decapitation and exsanguinated. The livers were removed and immediately placed in ice-cold TEDGP buffer [20 mM Tris-HCl, 1 mM Na_2EDTA, 1 mM dithiothreitol, 10% glycerol (w/v), and 0.5 mM phenylmethanesulfonyl fluoride, pH 7.4]. After the livers were minced, they were homogenized on ice with 5 mL of TEDGP buffer per gram wet weight of liver. The homogenate was centrifuged for 20 min at 10000*g* at 4 °C. The supernatant fraction was removed and centrifuged further at 100000*g* for 60 min at 4 °C. After careful aspiration of the fatty layer, the 100000*g* supernatant fraction was collected and used in the purification protocol described below.

Assay for Specific Binding. Protein fractions were assayed for specific [^{3}H]BP binding activity by using the HAP assay with slight modifications from that previously described (Gasiewicz & Neal, 1982). Aliquots containing 1 mg/mL or less of protein were incubated in the presence of [^{3}H]BP (10 nM) either with or without a 200-fold excess of cold BP. The total incubation volume was 0.5 mL in TEDGP buffer. After 60 min on ice, 0.5 mL of HAP slurry (3.3 g/50 mL of TEDGP) was added and the mixture allowed to incubate for an additional 30 min with occasional gentle mixing. Samples were centrifuged at 8000*g* for 20 s to pellet the HAP, and the supernatant solution was discarded. The HAP was washed 3 times with 1.0 mL of ice-cold 0.25% Tween 80 (v/v) in TEDGP. After the final wash, the HAP was resuspended and added to scintillation cocktail. The bound radioactivity was quantitated by liquid scintillation spectrometry.

Purification of Binding Protein. A Sepharose-CM ion-exchange column (1.5 × 30 cm) was equilibrated at 4 °C with TEDGP. The cytosolic fraction (100000*g* supernatant solution) was loaded under gravity flow (15 mL/h), and 7.5-mL fractions collected. During this step and subsequent steps, eluting protein was monitored by the absorbance at A_{280}. After sample loading, the column was washed with TEDGP until the A_{280} was less than 0.1. The eluant was assayed for specific binding activity, and the appropriate fractions were pooled.

The eluant from the Sepharose-CM column was concentrated by precipitation with 70% ammonium sulfate. Solid ammonium sulfate was added gradually with gentle stirring until completely dissolved (approximately 45–60 min). The solution was stirred an additional 15 min and then centrifuged at 10000*g* for 20 min to pellet the protein. The supernatant solution was discarded and the protein resuspended in a minimum volume (15–20 mL) of ice-cold TEDGP. The protein was "charged" by incubating with 1–2 μCi of [^{3}H]BP for 45–60 min.

A Sephacryl S-200 column (90 × 2.5 cm) was equilibrated with TEDGP at 4 °C. The charged ammonium sulfate fraction was loaded under gravity flow (15 mL/h), and 5-mL fractions were collected. Radioactivity was determined in each fraction. Active fractions were pooled and assayed for specific BP binding activity.

A phenyl-Sepharose column (2.6 × 10 cm) was washed with 100 mL of ice-cold 30% ethylene glycol (v/v) in TEDGP and then equilibrated with TEDGP alone. The combined radioactive fractions from the Sephacryl S-200 column were loaded onto the phenyl-Sepharose column under gravity flow (15 mL/h). Five-milliliter fractions were collected. After the sample was loaded, the column was washed with TEDGP until the A_{280} was less than 0.1. A 0.6% (w/v) CHAPS solution in TEDGP was then applied to the column. The protein fractions eluting with 0.6% CHAPS were combined and assayed for specific BP binding activity.

The active fractions from phenyl-Sepharose were loaded under gravity flow onto a DEAE-Sepharose column (2.5 × 30 cm) previously equilibrated at 4 °C with TEDGP. After the sample was loaded, the column was washed with TEDGP. A 250-mL 0–0.15 M NaCl gradient in TEDGP was applied to the column. Five-milliliter fractions were collected. Upon completion of the gradient, the column was washed with 0.5 M NaCl in TEDGP, pH 7.4. Fractions were assayed for radioactivity, combined, and assayed for specific BP binding activity.

The active fractions from the DEAE-Sepharose column were loaded onto an Affi-Gel Blue column (1.0 × 5 cm) previously equilibrated at 4 °C with TEDGP. After the sample was loaded, the column was washed with TEDGP, and 5-mL fractions were collected. A 0.5 M NaCl solution in TEDGP (50 mL) was applied to the Affi-Gel Blue column followed by TEDGP buffer with no salt. Finally, the binding protein was eluted with 0.6% CHAPS (w/v) solution in TEDGP. Fractions were assayed for radioactivity, combined, and assayed for specific binding activity.

SDS Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) was used to assess the purity of the binding protein eluting from the Affi-Gel Blue column. After electrophoresis, gels were fixed in methanol/acetic acid/water (40:10:50) and stained with Bio-Rad silver stain.

Determination of Sedimentation Coefficient. Aliquots (0.25 mL) of purified binding protein in TEDGP were layered onto a linear 5–20% sucrose gradient prepared in Beckman vertical rotor tubes. Protein standards (ribonuclease A, ovalbumin, bovine serum albumin, aldolase, and catalase) were also layered onto 5–20% linear sucrose gradients. The gradients were centrifuged for 2 h at 55000 rpm in a Beckman VTi 65 rotor (G_{av} = 275000*g*). After centrifugation, 150-μL fractions were collected and assayed for either radioactivity or A_{280}. The elution volume of the protein standards was plotted against the sedimentation coefficients, and a best-fit regression line was used to calculate the *s* value of the binding protein.

Determination of Stokes Radius. A Sephadex G-100 column (90 × 1.6 cm) was equilibrated at 4 °C with TEDGP. The column was calibrated by using protein standards (bovine serum albumin, ovalbumin, chymotrypsinogen A, ribonuclease A, and thyroglobulin). The molecular weight was calculated from the Stokes radius and the sedimentation coefficient (Siegel & Monty, 1966).

Construction of Cytochrome P-450c Clones. Our laboratory has isolated and sequenced the rat cytochrome P-450c gene (Hines et al., 1985). In addition, subclones have been prepared which contain different fragments of the gene. The construction of the various subclones is described in detail elsewhere (Hines et al., 1985). Briefly, pA8 was constructed by cloning a 5.5 kb *Eco*RI cytochrome P-450c gene fragment (+999–300 bp downstream from the gene) into the unique *Eco*RI site of pBR322. pMC13 was constructed by using pUC8 as a vector and cloning the *Bam*HI fragment (−824 to

+3446) from the cytochrome P-450c gene into the polylinker region of pUC8. pA9 was constructed again by using pUC8 as the vector and cloning the *Bam*HI/*Eco*RI fragment (−824 to +999) from the cytochrome P-450c gene into the polylinker region.

Radioactive Labeling of DNA. To investigate the interaction of the binding protein with DNA, total rat liver DNA and the various plasmids were labeled with deoxycytidine 5′-α-[^{35}S]thiotriphosphate (1300 Ci/mmol) by using the replacement synthesis method (O'Farrell et al., 1980). The various plasmids containing the fragments of the rat cytochrome P-450c gene were linearized by *Hind*III endonuclease digestion in TA buffer (33 mM Tris–acetate, 66 mM potassium acetate, 10 mM magnesium acetate, 100 μg/mL nuclease free bovine serum albumin, and 0.5 mM dithiothreitol, pH 7.9). After digestion, T4 DNA polymerase was added directly to the reaction mix to achieve a ratio of 1.5 units of enzyme/μg of DNA. After the exonuclease (Goulian et al., 1968) was allowed to act for 70 min at 37 °C, dATP, dGTP, and dTTP were added to 0.05 mM each together with 40 μCi of α-[^{35}S]thio-dCTP, and the incubation was continued for 60 min to allow the polymerase activity to fill in the digested ends. Unincorporated nucleotides were removed by using small Sephadex G-50 spin columns. Plasmids were extracted twice with equal volumes of buffer-saturated phenol, buffer-saturated PCI (phenol/chloroform/isoamyl alcohol, 49:49:1), and buffer-saturated ether.

Interaction of Binding Protein with DNA. The interaction of the binding protein with specific DNA was investigated by using the streptomycin assay (Spelsberg, 1983). The binding protein (1.5 μg) was incubated for 60 min at 4 °C with 100 ng of DNA in microfuge tubes containing 100 μL of binding buffer (25 mM Tris-HCl, 0.1 mM Na_2EDTA, 1 mM magnesium acetate, 0.1 mM dithiothreitol, and 0.15 M NaCl, pH 7.85). A 20-fold excess (w/w) of cold pBR327 was included in all incubations to facilitate precipitation of DNA. After 60 min, 20 μL of streptomycin [10 mg/mL (w/v) in distilled water] was added and the sample incubated at 4 °C for an additional 20 min to precipitate the DNA. Preliminary experiments in our laboratory have shown that concentrations of streptomycin up to 0.4% (w/v) do not inhibit the specific binding activity of this protein (data not shown). The incubation mixtures were centrifuged at 12000*g* for 10 min, and the supernatant solutions were discarded. The DNA was resuspended in 100 μL of binding buffer and applied to 25-mm nitrocellulose filters. The samples were washed 2 times with 0.25 mL of binding buffer under gentle filtration. The radioactivity bound to the filters was quantitated by liquid scintillation spectrometry.

Protein Determination. The amount of protein was determined with bovine serum albumin as a standard (Lowry et al., 1951). Alternatively, measurement of the ratio of the absorbance at 260 nm to that at 280 nm was used when the protein concentration was low (Warburg & Christian, 1941).

Liquid Scintillation Spectrometry. All samples were added to Hydrocount (American Scientific Products), and the radioactivity was quantitated by using a Packard Tri-Carb Series 4000 Minaxi scintillation counter. The efficiency of tritium counting was between 30% and 35%.

Results

The specific BP binding activity of this protein was assayed by using the hydroxylapatite assay with slight modifications (Gasiewicz & Neal, 1982). Other methods of assay such as charcoal–dextran or sucrose density gradient analysis were unreliable for accurate quantitation of specific binding activity when protein concentrations were very low, i.e., less than 300 μg of protein/mL.

Table I: Purification of Specific PAH Binding Proteins from Rat Liver

purification step	protein (mg)	sp act. (cpm × 10^{-3}/mg of protein)	x-fold purification: step	x-fold purification: overall	total act.
100000*g* homogenate	750	2.04	1	1	100
Sepharose-CM	465	5.54	2.72	2.72	168
Sephacryl S-200	99	31.37	5.66	15.38	203
phenyl-Sepharose	7	1086	34.6	533	497
DEAE-Sepharose	1.02	5095	4.69	2499	341
Affi-Gel Blue	0.125	12500	2.45	6130	102

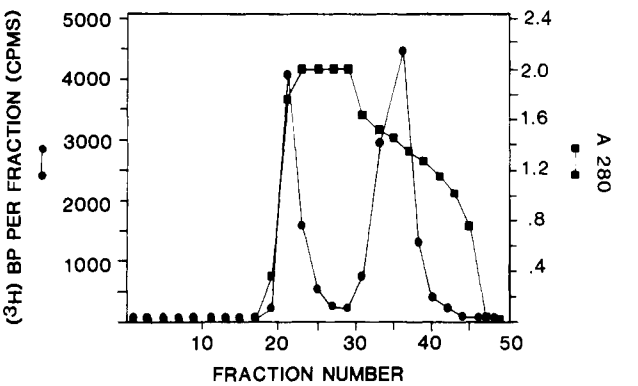

FIGURE 1: Elution profile from Sephacryl S-200. Cytosolic proteins (100000*g* supernatant fraction) were eluted from a Sepharose-CM column and concentrated by precipitation with 70% ammonium sulfate. The proteins were charged by incubation with 1–2 μCi of [^{3}H]BP and applied to a Sephacryl S-200 column (90 × 2.5 cm). The column was eluted with TEDGP, pH 7.4 (15 mL/h), and 5-mL fractions were collected. Absorbance at A_{280} (■) and radioactivity (●) were measured. The radioactive fractions were combined and assayed for specific binding activity.

Attempts to purify the binding protein have yielded several interesting observations. This protein exhibits an extreme hydrophobicity which may be understandable in view of its substrate specificity. This hydrophobicity leads to almost irreversible binding to hydrophobic matrices, especially when the protein is bound to a hydrophobic ligand such as BP. Unexpectedly, a high degree of irreversible binding and loss of activity were noted when cellulose matrices were used. Variable nonspecific losses of activity were also noted with some dextran matrices. In our experience, the Sepharose-based ion-exchange and gel permeation gels provided consistently higher recovery of specific binding activity.

Table I presents the results of a typical purification scheme. We elected to use a column procedure for the ion-exchange steps because of the ease of equilibration and the ability to monitor protein simultaneously. The purification (2.7-fold) and the yield from the Sepharose-CM column were similar to results previously reported in our laboratory (Tierney et al., 1983). The eluant from the Sepharose-CM column was concentrated by precipitation with 70% ammonium sulfate. After centrifugation at 10000*g* for 20 min, the protein was resuspended in fresh buffer and charged with 1–2 μCi of radiolabeled BP. The charging facilitated the identification of the binding protein as well as assisted in its stabilization. The charged protein was loaded onto a Sephacryl S-200 column and eluted under gravity flow (Figure 1). Two peaks of radioactivity were collected from the S-200 column. One peak eluted near the void volume of the column and demon-

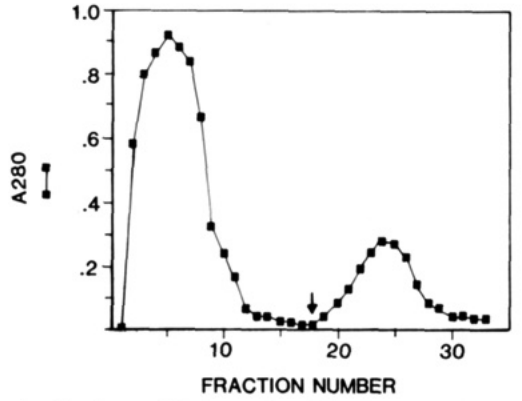

FIGURE 2: Elution profile from phenyl-Sepharose. The combined fractions from the Sephacryl S-200 column containing specific binding activity (tubes 30–40) were applied to a phenyl-Sepharose column (2.6 × 30 cm) previously washed with 100 mL of cold ethylene glycol (30% w/v) in TEDGP and equilibrated with TEDGP, pH 7.4. The column was washed with TEDGP (15 mL/h), and 5-mL fractions were collected. When the A_{280} was less than 0.1, a 0.6% CHAPS solution (w/v) in TEDGP, pH 7.4, was applied to the column (indicated by the arrow). Fractions containing protein eluting during the CHAPS wash (tubes 20–30) were combined and assayed for specific binding activity. Specific binding activity was present only in those fractions eluting with CHAPS.

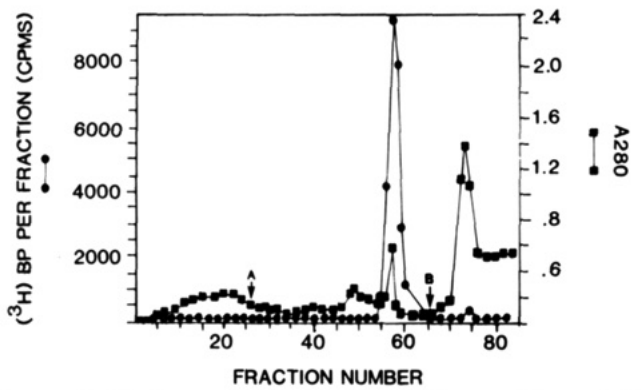

FIGURE 3: Elution profile from DEAE-Sepharose. The pooled fractions from the phenyl-Sepharose column were applied to a DEAE-Sepharose column (2.5 × 30 cm). The column was washed with TEDGP, pH 7.4, and 5-mL fractions were collected. The A_{280} (■) and radioactivity (●) were measured. A 250-mL 0–0.15 M NaCl gradient in TEDGP was applied to the column (indicated by arrow A). Upon completion of the gradient, a 0.5 M NaCl solution in TEDGP was applied to the column (indicated by arrow B). Radioactive fractions were pooled and assayed for specific binding activity.

strated only nonsaturable BP binding. A second peak between fractions 30 and 40 contained the specific BP binding activity. This step yielded only a moderate purification (5.6-fold) but removed a considerable amount of contaminating protein.

The active fractions from the Sephacryl S-200 column were pooled and loaded onto a phenyl-Sepharose column. After the column was washed, the binding protein was eluted from the column by using a 0.6% CHAPS solution in TEDGP buffer (Figure 2). CHAPS, a zwitterionic detergent (Hjelmeland, 1980), at concentrations up to 1% did not affect the specific binding to BP (data not shown). Elution from phenyl-Sepharose using CHAPS yielded a high degree of purification (34.6-fold).

It is interesting to note that during the early purification steps an apparent increase in total yield is noted. We believe this to be due to the removal of inhibitory factors (competitive binding proteins such as the 9S protein) during the early stages of the purification. This phenomenon is discussed in a recent study (Denison et al., 1984).

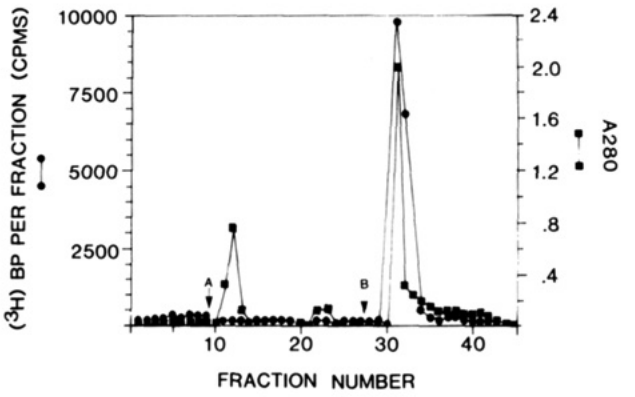

FIGURE 4: Elution profile from Affi-Gel Blue. The combined fractions from DEAE-Sepharose (tubes 55–60) were applied to an Affi-Gel Blue column (1.0 × 5 cm). The column was washed with TEDGP, pH 7.4 (15 mL/h). Five-milliliter fractions were collected, and the A_{280} (■) and radioactivity (●) were determined. Fifty milliliters of a 0.5 M NaCl solution in TEDGP, pH 7.4, was applied to the column (indicated by arrow A). After reequilibration of the column with TEDGP buffer, a 0.6% CHAPS solution (w/v) in TEDGP, pH 7.4, was applied (indicated by arrow B). Radioactive fractions were pooled and assayed for specific binding activity.

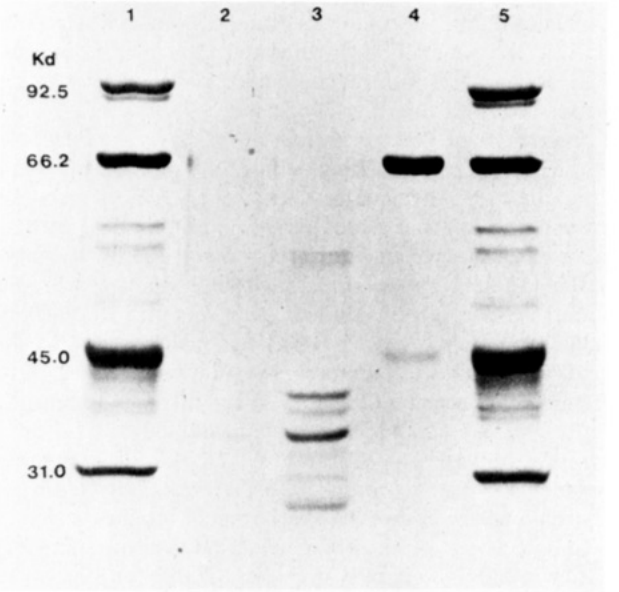

FIGURE 5: SDS–polyacrylamide gel electrophoresis. After elution from the Affi-Gel Blue column, sample and standards were applied, and electrophoresis was carried out in 10% linear polyacrylamide gels. Lanes 1 and 5 are molecular weight (in parentheses) standards including phosphorylase *b* (92 500), BSA (66 200), ovalbumin (45 000), and carbonic anhydrase (31 000). Lane 2, Hydroxylapatite eluent (<0.5 μg); lane 3, Affi-Gel Blue eluent (2.0 μg); lane 4, BSA and ovalbumin (1.0 μg of each).

The protein eluted from phenyl-Sepharose with 0.6% CHAPS was loaded onto a DEAE-Sepharose column (Figure 3). The presence of CHAPS did not interfere with the performance of this ion-exchange chromatographic step. During the application of a NaCl gradient, the binding protein was eluted with 0.07 M salt. The column was washed with 0.5 M NaCl to elute any remaining protein. Only the protein eluted under low-salt conditions displayed specific binding activity. The relative purification of this step was 4.7-fold.

Finally, the active fractions from the DEAE-Sepharose were loaded onto an Affi-Gel Blue column and washed with TEDGP buffer (Figure 4). When the column was washed with 0.5 M NaCl, a peak of protein was eluted. Analysis of these fractions showed there was neither radioactivity nor specific BP binding activity. The binding protein, however, could be

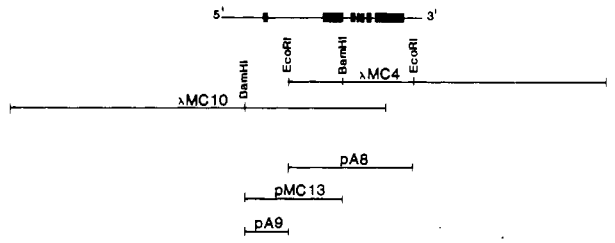

FIGURE 6: Rat cytochrome P-450c gene. The rat cytochrome P-450c gene is approximately 6.7 kb in length (Hines et al., 1985). The gene contains seven exons (indicated by shaded boxes) and six introns. The amino and carboxy termini are located in exon 2 and exon 7, respectively. Two clones (λMC4 and λMC10) were isolated from rat genomic libraries and collectively contained the entire gene for rat cytochrome P-450c. Subclones containing portions of the rat cytochrome P-450c gene (pA8, pMC13, and pA9) were prepared in plasmid and phage vectors. The information contained in each subclone is shown in the figure.

eluted with 0.6% CHAPS in TEDGP buffer. The recovery of protein was low in this step, but a purification of 2.5-fold was achieved.

The fractions containing specific binding activity were subjected to SDS–polyacrylamide gel electrophoresis on 10% linear gels and stained with silver (Figure 5, lane 3). Four major bands are presented with molecular weights of 28 000, 34 000, 36 000, and 39 000. Minor bands are present with molecular weights of 31 000, 33 000, 56 000, and 57 000. We consistently find the two strong bands at 34 000 and 39 000 and believe that one or both of these represent our PAH binding protein. We have recently loaded our Affi-Gel Blue fractions onto a small hydroxylapatite column and eluted our protein using a 0.0–0.25 M phosphate gradient. The radioactive fractions were combined and concentrated. This fraction is represented in lane 2 of Figure 5. A single, faint band was detected with a molecular weight of 34 000. However, we hesitate to make further conclusions on the purity of this fraction because of the small amount of sample loaded onto this gel. The presence of the minor bands is variable, and we believed they represent other strongly hydrophobic proteins which copurify with the PAH binding protein. We have attempted to perform electrophoresis of this protein under nonreducing and nondenaturing conditions but were not successful.

The determination of the sedimentation coefficient for the purified protein using sucrose gradient analysis yielded a value of 3.3 S. This is slightly lower than has been previously reported but may be due to a more highly purified protein. Using calibrated Sephadex G-100 chromatography, a Stokes radius of 2.75 nm was determined. An apparent molecular weight of approximately 40 000 was calculated from the Stokes radius and the sedimentation coefficient (Siegel & Monty, 1966). These latter values are consistent with previously reported results for this protein (Tierney et al., 1980, 1983).

Figure 6 depicts the rat cytochrome P-450c gene and the information contained in the different subclones. In this figure, the seven exons are depicted by the solid boxes. pA8 has approximately 5.4 kbp of rat genomic information and contains all of the coding information for the rat cytochrome P-450c gene as well as the 3′ half of intron 1. pMC13 contains approximately a 4.3 kbp insert including exon 1, intron 1, part of exon 2, and also 824 bp of 5′ information. pA9 contains 1.9 kbp of cloned sequences including exon 1, the 5′ half of intron 1, and also 824 bp of upstream information.

The ability of the purified binding protein to interact with either total rat liver DNA or plasmid DNA containing various portions of the rat cytochrome P-450c gene was investigated

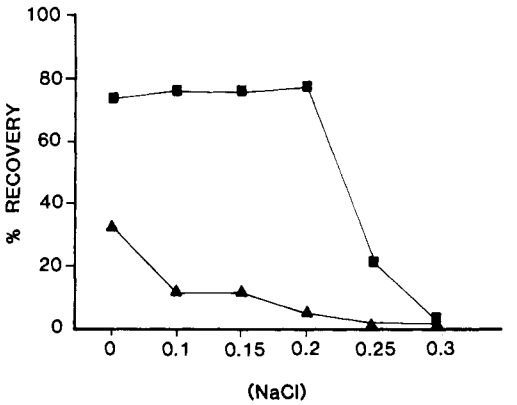

FIGURE 7: Effect of ionic strength on the recovery of DNA. To assess the effect of ionic strength on the integrity of the streptomycin–DNA complex (■), receptor protein (1.5 μg), radiolabeled pUC8 (100 ng), and pBR327 (2 μg) were incubated at 4 °C for 60 min in 100 μL of binding buffer (described under Materials and Methods). Twenty microliters of streptomycin (10 mg/mL in distilled water) was added, and samples were incubated an additional 20 min. After centrifugation for 10 min at 12000*g*, the supernatant was removed carefully and the DNA pellet resuspended in 100 μL of binding buffer. The resuspended DNA was added to cocktail and the radioactivity determined by liquid scintillation spectrometry. The concentration of NaCl (abscissa) is given in molar. Values represent the mean of two determinations and are expressed as the percentage of radioactivity recovered vs. radioactivity added. To assess the effect of ionic strength on the integrity of the DNA–receptor complex (▲), receptor protein and DNA were incubated as described above. After resuspension, the DNA was filtered through nitrocellulose filters (25 mm, 0.45 μm). The filters were washed twice with 0.25 mL of binding buffer (with the appropriate molarity of NaCl) and dried. The filters were added to scintillation cocktail, and the radioactivity was determined by liquid scintillation spectrometry. Values represent the mean of four determinations and are expressed as the percentage of radioactivity recovered vs. the radioactivity added.

by using the streptomycin assay (Spelsberg, 1983). This assay selectively precipitates DNA or DNA–protein complexes. To assure the specificity of the protein–DNA complex, preliminary studies were conducted to maximize the stringency. Two factors must be considered. First, concentrations of high salt will dissociate the streptomycin–DNA complex which would lead to poor precipitation and the subsequent low recovery of DNA. Second, concentrations of high salt may dissociate even a specific protein–DNA complex which would lead to less retention of DNA by the nitrocellulose filters and to a loss of sensitivity.

Figure 7 shows the results of the recovery of DNA and the binding of protein–DNA complexes to nitrocellulose filters as a function of NaCl concentration. Concentrations of NaCl above 0.25 M were found to dissociate the streptomycin–DNA complex and lead to a significant loss of DNA. Also, when the precipitated protein–DNA complexes were applied to nitrocellulose filters, a reduction in the recovery of DNA was observed between 0 and 0.1 M NaCl due to the inhibition of nonspecific protein–DNA complexes. A plateau of recovery, due to specific protein–DNA complexes, is observed between 0.1 and 0.15 M NaCl but falls off even further above 0.15 M NaCl, indicating a loss of the protein–DNA complex. Therefore, a concentration of 0.15 M NaCl was chosen for the investigation of the interaction of the binding protein with DNA.

Table II summarizes the results of the interaction of the binding protein with either total rat liver DNA or the various subclones containing different portions of the rat cytochrome P-450c gene. Incubations containing receptor–[^{3}H]BP in the absence of DNA showed little or no radioactivity on the nitrocellulose filter. These data confirm that the protein–ligand

Table II: Interaction of Binding Protein with ^{35}S-Labeled DNA[a]

	pA9	pA9	pMC13	pUC8	TRL
REC	0.1 ± 0.05	0.27 ± 0.1	0.31 ± 0.3	0.3 ± 0.4	0.04 ± 0.03
DNA	2.8 ± 1.24	6.1 ± 2.5	3.69 ± 2.0	2.52 ± 0.5	3.31 ± 0.3
REC + DNA	8.2 ± 1.06 (0)[b]	23.5 ± 6.2 (82.65%)	22.3 ± 6.2 (73.54%)	12.0 ± 1.9 (0)	25.9 ± 8.6 (100%)
BSA + DNA	8.3	10.8 ± 0.3	10.5 ± 0.6	8.48 ± 1.7	15.4 ± 1.2
100X XS DNA		5.98 ± 1.1	8.4 ± 1.6		
200X XS DNA		1.98 ± 2.4	2.9 ± 0.9		

[a] Incubations contained receptor alone (REC), DNA alone (DNA), receptor and DNA (REC + DNA), bovine serum albumin and DNA (BSA + DNA), or receptor and DNA with a 100-fold excess (100X XS DNA) or 200-fold excess (200X XS DNA) of unlabeled DNA. Values are the mean ± standard deviations of a least four determinations and are expressed as percents (radioactivity recovered on nitrocellulose filters vs. radioactivity added to incubations). [b] Values in parentheses are corrected for background (pUC8 binding) and expressed as a percent of total rat liver (TRL) binding.

complex alone is not precipitated by streptomycin. Incubations with ^{35}S-labeled DNA, in the absence of the receptor–ligand complex, showed minimal binding to the nitrocellulose filters. Incubation of the receptor–ligand complex with radiolabeled DNA resulted in a 3–4-fold increase in the amount of radioactivity retained on the nitrocellulose filter. Two subclones, pA9 and pMC13, appeared to bind the receptor–ligand complex as well as total rat liver DNA (positive control). In contrast, pA8 and pUC8 (a plasmid vector control) were not bound as avidly by the binding protein. Incubations of the DNA from various subclones with BSA, a protein which interacts nonspecifically with PAH, also resulted in background levels of binding. The interaction of pA9 and pMC13 DNA with the protein was saturable as demonstrated by the displacement of labeled DNA by a 200- or a 100-fold excess of unlabeled pA9 or pMC13, respectively. Binding to total rat liver DNA was also inhibited by a 100-fold excess of pA9 (data not shown).

Discussion

In this study, we have described a simplified protocol for the preparation of 6000-fold-purified "4S" polycyclic aromatic hydrocarbon binding protein. In addition, we have investigated the potential role of this protein in the regulation of rat cytochrome P-450c expression. Specifically, we have examined the ability of the "4S" protein to bind various plasmid DNA containing fragments of the rat cytochrome P-450c gene. The results of our studies indicate that the "4S" protein interacts specifically with plasmids containing primarily noncoding information 5′ to exon 2 (intron 1, exon 1, and 824 bp 5′ to the promoter). This interaction is similar to that described for certain steroid hormones. It has recently been demonstrated that the glucocorticoid receptor–ligand complex recognizes specific DNA sequences and binds to regulatory elements between −72 and −192 bp upstream from the main transcription initiation site on the mouse mammary tumor virus long terminal repeat region (Scheidereit & Beato, 1984). Interestingly, investigators have recently reported a binding region for TCDD between −1580 and −1310 bp 5′ to the promoter region on the mouse cytochrome P_1-450 gene (Jones et al., 1985).

The role of the 9S binding protein in the regulation of AHH activity by TCDD has been investigated in a number of studies using wild-type and variant mouse hepatoma cell lines (Miller et al., 1983; Israel & Whitlock, 1983; Whitlock & Galeazzi, 1984; Jones et al., 1984, 1985). In addition, investigators have examined the binding of BP and DBA to the 9S binding protein in mouse and rat hepatic cytosols (Okey et al., 1984). The latter investigation emphasized several important observations: (1) The 9S protein was not apparent in the hepatic cytosols of the genetically responsive C57BL/6J (B6) mice when BP was used as the ligand. BP bound only to the "4S" region in B6 cytosol. Despite the apparent inability of BP to bind to the 9S protein in B6 mice, BP is quite efficacious in its ability to induce AHH activity in this strain of mice. Since BP induces AHH activity in B6 mice in the apparent absence of the 9S receptor, conclusions that only the 9S receptor mediates AHH induction based solely on the genetic segregation of the 9S receptor species may not be accurate. (2) A second important observation is that TCDD is not a good ligand for the "4S" receptor. This was corroborated by Okey et al. (1984) and by others (Zytkovicz, 1982; Collins & Marletta, 1984). Clearly, the majority of binding by the various PAHs in the B6 mouse liver cytosol was to a "4S" protein. Thus, conclusions by Okey et al. (1984) and others on the regulatory role of the "4S" protein based solely on the inability of TCDD to saturate PAH binding to this protein may be misdirected. Indeed, a number of investigators have demonstrated that PAH binding to the "4S" binding protein is saturable by a 100–200-fold excess of unlabeled PAH ligands (Zytkovicz, 1982; Tierney et al., 1983; Collins & Marletta, 1984).

Our results indicate that the "4S" binding protein is able to interact in a specific and saturable manner with certain elements of the rat cytochrome P-450c gene. Now that we have obtained a purified binding protein, "footprinting" studies will be undertaken to more clearly define the specific binding regions on our cytochrome P-450c subclones. These studies will hopefully help to clarify the role of the "4S" binding protein in the transregulation of rat cytochrome P-450c expression. However, taking into consideration the data presented here, as well as those recently reported (Jones et al., 1985), it is most likely that both the "4S" and 9S proteins are involved but perhaps through different mechanisms and in response to different classes of inducing agents.

Registry No. BP, 50-32-8.

References

Bresnick, E., Liebelt, R. A., Stevenson, J. G., & Madix, J. C. (1967) *Cancer Res. 27*, 462–468.

Brodie, B. B., Reid, W. D., Cho, A. K., Sipes, G., Krisha, G., & Gillette, J. R. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 160–164.

Collins, S., & Marletta, M. A. (1984) *Mol. Pharmacol. 26*, 353–359.

Conney, A. H. (1967) *Pharmacol. Rev. 19*, 317–366.

Denison, M. S., Fine, J., & Wilkinson, C. F. (1984) *Anal. Biochem. 142*, 28–36.

Gasiewicz, T. A., & Neal, R. A. (1982) *Anal. Biochem. 124*, 1–11.

Gillette, J. R., Davis, D. C., & Sesame, H. A. (1972) *Annu. Rev. Pharmacol. 12*, 57–84.

Goulian, M., Lucas, Z. J., & Kornberg, A. (1968) *J. Biol. Chem. 243*, 627–638.

Guenthner, T. M., & Nebert, D. W. (1977) *J. Biol. Chem. 252*, 8981–8989.

Heintz, N. H., Tierney, B., Bresnick, E., & Schaeffer, W. I. (1981) *Cancer Res. 41*, 1794–1802.
Hines, R. N., Levy, J. B., Conrad, R. D., Iversen, P. L., Shen, M.-L., Renli, A., & Bresnick, E. (1985) *Arch. Biochem. Biophys. 237*, 465–476.
Hjelmeland, L. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6369–6370.
Israel, D. I., & Whitlock, J. P. (1983) *J. Biol. Chem. 258*, 10390–10394.
Jones, P. B. C., Miller, A. G., Israel, D. I., Galeazzi, D. R., & Whitlock, J. P. (1984) *J. Biol. Chem. 259*, 12357–12363.
Jones, P. B. C., Galeazzi, D. R., Fisher, J. M., & Whitlock, J. P. (1985) *Science (Washington, D.C.) 227*, 1499–1502.
Kimura, S., Gonzalez, F. J., & Nebert, D. W. (1984) *J. Biol. Chem. 259*, 10705–10713.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Miller, A. G., Israel, D. I., & Whitlock, J. P. (1983) *J. Biol. Chem. 258*, 3523–3527.
Miller, E. C., & Miller, J. A. (1966) *Pharmacol. Rev. 18*, 805–838.
Nebert, D. W., Eisen, H. J., & Hankinson, O. (1984) *Biochem. Pharmacol. 33*, 917–924.
Okey, A. B., Bondy, G. P., Mason, M. E., Kahl, G. F., Eisen, H. J., Guenthner, T. M., & Nebert, D. W. (1979) *J. Biol. Chem. 254*, 11636–11648.
Okey, A. B., Dube, A. W., & Vella, L. M. (1984) *Cancer Res. 44*, 1426–1432.
Poland, A., Glover, E., & Kende, A. S. (1976) *J. Biol. Chem. 251*, 4936–4946.
Ryan, D. E., Thomas, D. E., Koreniowski, D., & Levin, W. (1979) *J. Biol. Chem. 254*, 1365–1379.
Scheidereit, C., & Beato, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3029–3033.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.
Spelsberg, T. C. (1983) *Biochemistry 22*, 13–21.
Tierney, B., Weaver, D., Heintz, N., Schaeffer, W. I., & Bresnick, E. (1980) *Arch. Biochem. Biophys. 200*, 513–523.
Tierney, B., Munzer, S., & Bresnick, E. (1983) *Arch. Biochem. Biophys. 225*, 826–845.
Warburg, O., & Christian, W. (1941) *Biochem. Z. 310*, 384.
Weisburger, J. H., & Weisburger, E. K. (1973) *Pharmacol. Rev. 25*, 1–66.
Whitlock, J. P., & Galeazzi, D. R. (1984) *J. Biol. Chem. 259*, 980–985.
Zytkovich, T. H. (1982) *Cancer Res. 42*, 4387–4393.

Cytochrome *c* Oxidase from the Slime Mold *Dictyostelium discoideum*: Purification and Characterization†

Roberto Bisson,* Giampietro Schiavo, and Emanuele Papini
CNR Center for the Study of the Physiology of Mitochondria and Laboratory of Molecular Biology and Pathology, Institute of General Pathology, University of Padua, 35100 Padova, Italy
Received April 24, 1985

ABSTRACT: Cytochrome *c* oxidase was purified from growing cells of the slime mold *Dictyostelium discoideum* by a procedure based on hydrophobic and affinity chromatography. A highly pure (13.4–15 nmol of heme *a*/mg of protein) and active (turnover number = 280–330 s^{-1}, when assayed polarographically with the slime mold cytochrome *c*) enzyme preparation was obtained. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, under conditions where the 12 polypeptide components of the bovine enzyme are resolved, shows that the amoeba oxidase consists of six subunits with molecular masses of 55, 29.5, 19, 13, 11, and 5.7 kDa. A polypeptide with the characteristics of the eukaryotic subunit III is missing, and *N*,*N*′-dicyclohexylcarbodiimide, a specific reagent for this component, labels subunit I. Under controlled conditions and even at physiological pH, the single subunit present at M_r <10000 can be selectively removed from the complex. Hydrophobic photolabeling suggests that with the mitochondrial subunits I and II only subunit IV among the nuclear coded polypeptides is in contact with lipids.

One of the most intricate aspects of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, concerns the number and function of its subunits. In higher eukaryotes up to 12–13 polypeptides are associated in the protein complex while, in contrast, only one to three of them are found in bacterial organisms where they seem sufficient to carry out the known enzyme functions (Buse et al., 1983; Ludwig, 1980; Yoshida et al., 1984). These observations are used to support growing biochemical evidences indicating that subunits I and II are the catalytic components of oxidase; the two polypeptides, in fact, appear to contain the prosthetic groups and bind the substrate (Bisson et al., 1978, 1982; Winter et al., 1980; Darley-Usmar et al., 1981; Capaldi et al., 1983; Suarez et al., 1984). Subunit III, the third largest enzyme component coded for by mDNA, has been implicated in the proton-pumping process although much controversy still exists concerning its specific function (Casey et al., 1980; Prochaska et al., 1981; Sarti et al., 1985). The role of the remaining nuclear coded polypeptides remains obscure, but their importance has been recently emphasized by the sequence homologies found in oxidases from different unrelated sources (Gregor & Tsugita, 1982; Power et al., 1984a) and by the discovery of tissue-specific isoenzymes (Kadenbach, 1983).

Although many recent studies have been focused on bacterial oxidases because of their structural simplicity, investigations on the enzyme of suitable eukaryotic sources may offer new insights on the possible roles of the cytoplasmic subunits

† This paper is dedicated to the memory of Robert P. Casey.
* Address correspondence to this author.

0006-2960/85/0424-7845$01.50/0