Vol. 104, No. 3, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 11, 1982 Pages 1093-1101

BINDING OF BENZO(A)PYRENE TO HEPATIC CYTOSOLIC PROTEIN ENHANCES ITS MICROSOMAL OXIDATION

Rakesh Dixit, David R. Bickers and Hasan Mukhtar*

Department of Dermatology, Case Western Reserve University,
University Hospitals of Cleveland and
Veterans Administration Medical Center
Cleveland, Ohio 44106

Received January 4, 1982

Sephadex G-100 gel permeation chromatography of rat liver cytosol saturated with ^{14}C-benzo(a)pyrene (BP) resulted in two peaks of protein bound radioactivity. Glutathione-S-transferase (GST) activity (towards 1-chloro 2,4-dinitrobenzene as substrate) was eluted as a single major peak which coincided with one peak of protein bound BP. Oxidation of protein bound BP (GST rich fractions) by microsomes from control or 3-methylcholanthrene treated rats was significantly enhanced as compared to ethanol suspended BP. The formation of oxidized products from the protein-bound BP was dependent on incubation time and microsomal protein concentration, required NADPH and was inhibited by monooxygenase inhibitors α-napthoflavone, 1-benzylimidazole, metyrapone and SKF 525A. Coemergence of BP binding-protein with GST suggests that the soluble protein could be one of the glutathione-S-transferases.

INTRODUCTION

Certain lipophilic carcinogens including PAHs are metabolized by the membrane-bound cytochrome P-450 dependent MFO system into a variety of intermediates which subsequently may be converted into electrophilic metabolites by one or several enzymes (1-3). According to current concepts the intermediate electrophilic metabolites of PAHs can either undergo rearrangement to phenols, can be conjugated with glutathione, or can bind to cellular macromolecules to initiate carcinogenesis (4-6). The enzymes involved in the metabolic activation of PAH carcinogens are membrane bound and are predominantly localized in the endoplasmic reticulum. Since the lipid soluble carcinogens

Abbreviations used: BP, benzo(a)pyrene; CDNB, 1-chloro-2,4-dinitrobenzene; PAH, polycyclic aromatic hydrocarbons; 3-MC, 3-methylcholanthrene; GST, glutathione-S-transferase; ER, endoplasmic reticulum; MFO, mixed function oxidase.

*To whom all correspondence should be addressed

0006-291X/82/031093-09$01.00/0

have a partition coefficient favourable to phospholipid bilayers it is reasonable to assume that, they tend to concentrate in the lipid-rich components of the ER (7). Such a phenomenon could result in blockage of the transport of these lipophilic carcinogens from one segment of the ER to another and to the site of MFOs action which is thought to be in the aqueous environment of the membrane within the ER. It is feasible that certain proteins with adequate hydrophobic binding sites in the cytosol may facilitate the transport of the lipophilic carcinogens such as benzo(a)pyrene, a ubiquitous environmental pollutant, to the site of MFO action. Although several carcinogen binding proteins including ligandin and azo dye-binding proteins have been demonstrated in the cytosol of many target tissues (8-10), the role of these proteins in the transport of lipid soluble carcinogens to the site of MFO action remains to be understood. Evidence is presented in this paper which indicates that binding of BP to a protein in rat liver cytosol facilitates its oxidation by microsomal enzymes.

MATERIALS AND METHODS

Animals and preparation of cytosol: Male Sprague-Dawley rats (160-180 g) were used in the present study. Animals were killed by cervical dislocation and livers were homogenized in 4 volumes of chilled 0.1 M phosphate buffer containing 0.15 M KCl, pH 7.4. Cytosols (100,000 g supernatants) were prepared according to conventional techniques (11). The cytosols were treated with activated charcoal (20 mg/ml) for 30 minutes and were then dialyzed overnight against excess of 0.01 M tris-HCl buffer, pH 8.2.

Preparation of cytosolic protein bound BP: [7-10-] ^{14}C-benzo(a)pyrene (specific activity 21.7 mCi/mMol in toluene) was purchased from Amersham Searle, Ill. 30 µCi of radiolabelled BP (1380 nmole) was evaporated to dryness under nitrogen and was suspended in 100 µl of ethanol. The purity of the BP as monitored by high pressure liquid chromatography was approximately 99%. 130 ml of the cytosol was mixed with this BP solution and the mixture was gently shaken at 4°C for 24 hours. In order to avoid any autooxidation of BP, all glassware was covered with aluminum foil.

Gel filtration chromatography and isolation of BP bound protein: 50 ml of ^{14}C-BP saturated cytosol was applied on top of a column (100 x 2.5 cm) containing Sephadex G-100 equilibrated and mobilized with 0.01 M tris-HCl, pH 8.2. Fractions (1.5 ml) were collected at a flow rate of 1.5 ml/min.

Determination of radioactivity and protein: 40 µl aliquots from each fraction were taken in a vial to which 10 ml of Scintiverse (Fisher Scientific CO) was added. Radioactivity was quantitated in a Packard TriCarb liquid Scintillation Spectrometer (model 3375) with a counting efficiency of 90%. C.P.M. were converted to D.P.M. using quench correction. The protein in each fraction was determined by the method of Lowry et al. (12).

Glutathione-S-transferase assay: GST activity in each eluted fraction was determined towards 1-chloro 2,4-dinitrobenzene as substrate according to Habig et al. (13).

Microsomal monooxygenase catalyzed oxidation of cytosolic protein-bound BP (Cp-BP): Microsomes from control or 3-MC pretreated rats were prepared according to

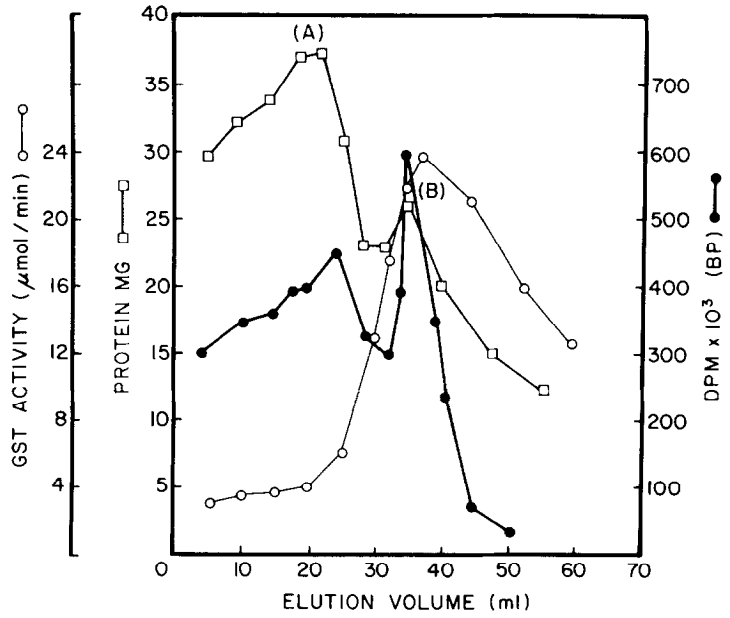

Fig 1. Elution profile of BP bound cytosolic protein from Sephadex G-100 gel: 50 ml of rat liver cytosol saturated with ^{14}C-BP was applied on the top of column (100 x 2.5 cm) of Sephadex G-100. The column was preequilibrated with 0.01 M tris HCl buffer, pH 8.2. BP-bound proteins were eluted with equilibrating buffer. Fractions (1.5 ml) were collected with a flow rate of 1.5 ml/min. Each fraction was analyzed for radioactivity (●—●), protein (□—□) and glutathione-S-transferase activity (○—○) as described in Methods.

established procedures (11). Cytosolic protein-bound BP radioactivity present in each of the peak fractions (Fig. 1) was pooled and was designated as Cp-BP (A) or Cp-BP (B). The procedure for microsomal oxidation of BP was adapted from the radioactive aryl hydrocarbon monooxygenase assay of DePierre et al. (14) and modified as follows. The reaction mixture for microsomal oxidation of BP or Cp-BP in a final volume of 1.0 ml contained 100 μ mole potassium phosphate buffer, pH 7.4, 1.2 μ mole NADPH and microsomal protein (0.1 - 0.4 mg). The reaction was started by the addition of 100 μl of Cp-BP or BP in 20 μl ethanol (particulate BP). The contents of the tubes under dark were incubated for 10 min. at 37°C after which the reaction was terminated by the addition of 1.0 ml of 0.5N NaOH in 80% ethanol. Unmetabolized BP was removed from the reaction products by extracting the mixture three times with 3.4 ml of n-hexane. Oxidation products were quantified by counting an aliquot of the aqueous alkaline layer.

RESULTS

Elution profile of BP - binding proteins: Identity of one of the proteins with GST.

The elution profile of BP binding proteins present in rat liver cytosol saturated with BP, on Sephadex G-100 gel is depicted in Figure 1. It is apparent that ^{14}C-BP was eluted in bound form with proteins at two major peaks. From the molecular weight analysis of protein, it is evident that the first peak of BP radioactivity is associated with high molecular weight proteins and the second one with proteins having a molecular weight in the range of 40,000 - 50,000. Each eluted fraction was analyzed for GST

Table 1

Oxidation of cytosolic protein bound benzo(a)pyrene by microsomal monooxygenase of control or 3-methylcholanthrene pretreated rats.

Complete = 1 ml incubation mixture containing 0.1 M phosphate buffer, pH 7.4, microsomal protein (0.1 - 0.4 mg) and NADPH (1.2 μ mole).

Assay conditions	Oxidized products DPM x 10^2/10 min/mg microsomal protein	
	control	3-MC
Complete + ^{14}C-BP (a)	231	512
Complete + ^{14}C-BP (b)	114	232
Complete + Cp-BP(B) (1.77 mg protein)	366	1218
Complete + Cp-BP(B) (0.88 mg protein)	205	656
Complete + ^{14}C-BP + albumin (1.77 mg)	222	502
Complete + ^{14}C-BP + boiled Cp-BP(B) (1.77 mg)	212	501
Complete - MIC + Cp-BP(B) (1.77 mg)	18	N.D

^{14}C-BP a,b indicate BP radioactivity present in bound form with 1.77 mg and 0.88 mg cytosolic protein respectively of Cp-BP (B).

MIC = microsomes. N.D. = not done

activity towards CDNB as substrate. As shown in figure 1, GST activity was eluted in a single major peak where a major fraction of second peak of protein bound BP radioactivity was also emerged. The total recovery of radioactivity from Sephadex G-100 was 60-80% of the total incubated BP.

Characterization of microsomal oxidation of cytosolic protein bound BP:

Oxidation of Cp-BP(B) by control or 3-MC pretreated microsomal monooxygenases is shown in Table 1. Cytosolic protein binding of BP resulted in a 60-80% increase in the oxidation product formation of BP as compared to oxidation of particulate BP. 3-MC treated microsomal preparations resulted in a 120-180% higher product formation from Cp-BP(B) when compared to oxidation of particulate BP. The replacement of Cp-BP(B) by an equivalent albumin concentration or boiled Cp-BP(B) had no enhancing effect on the microsomal oxidation of BP. No appreciable formation of oxidized products from Cp-BP(B) was noticed in the absence of microsomes or NADPH. Fig. 2 depicts the microsomal protein dependence of oxidation of Cp-BP(B). The oxidative product formation from Cp-BP(B) increased linearly with protein concentration and was

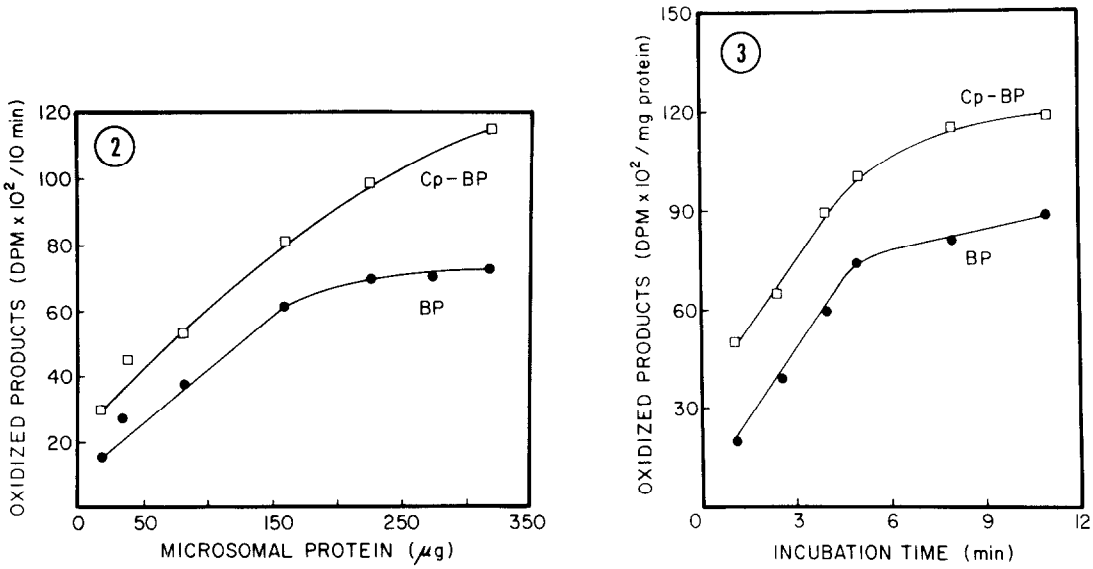

Fig 2. Dependence on protein concentration of oxidation of cytosolic protein bound BP (Cp-BP(B)) by microsomes: Complete incubation mixture in a final volume of 1.0 ml contained in addition to other reactants (see methods) desired concentration of microsomes. Reaction was initiated by the addition of ^{14}C-BP suspension in 20 μl ethanol or by the addition of 100 μl of Cp-BP (B) (1.17 mg protein). For other details see methods.

Fig. 3. Dependence on incubation time of oxidation of cytosolic protein bound BP (Cp-BP(B)) by microsomes: Reaction was intitiated by the addition of ^{14}C-BP suspension in 20 μl acetone or by the addition of 100 μl of Cp-BP(B) and was terminated at the times indicated.

significantly higher than the oxidation of particulate BP. The time dependent oxidation of Cp-BP(B) is shown in Fig. 3. The formation of products from Cp-BP(B) was linear up to 10 min. of incubation and was higher than when particulate BP was used. The cofactor requirements for the oxidation of Cp-BP(B) are presented in Table 2. The oxidation of Cp-BP(B) was dependent on NADPH. NADH served as a poor electron donor, however product formation was increased in a additive manner in the presence of NADPH and NADH. The effect of monooxygenase inhibitors added in vitro on the oxidation of Cp-BP(B) is shown in Table 3. It is evident that the oxidation of Cp-BP(B) was inhibited by all the four MFO inhibitors such as α-napthoflavone, 1-benzylimidazole, metyrapone and SKF-525A. α -Napthoflavone was highly inhibitory for the oxidation of both Cp-BP(B) or particulate BP.

The microsomal oxidation of Cp-BP(A) is recorded in Table 4. Oxidation of BP bound to protein peak A did not result in any enhancement of product formation when compared to the oxidation of particulate BP.

Table 2

Cofactor dependence for microsomal monooxygenase catalyzed oxidation of protein bound benzo(a)pyrene.

Complete = 1 ml incubation mixture containing 0.1 M phosphate buffer, pH 7.4, and microsomal protein (0.32 mg).

Incubation conditions	Oxidized products DPM x 10^2/10 min/mg microsomal protein with BP	with Cp-BP(B)
Complete	18	19
Complete + NADPH (0.6 μ mole)	176	286
Complete + NADPH (1.2 μ mole)	241	410
Complete + NADH (0.7 μ mole)	62	96
Complete + NADH (1.4 μ mole)	76	111
Complete + NADH (0.6 μ mole) + NADPH (0.7 μ mole)	241	331

DISCUSSION

The present study demonstrates the presence of two BP binding proteins in rat liver cytosol. From the elution profile of the BP binding proteins and molecular weight analysis it can be seen that BP is significantly bound to (a) high molecular weight proteins and (b) to proteins with a molecular weight in the range of 40,000 - 50,000. The

Table 3

Effect of monooxygenase inhibitors on microsomal oxidation of protein bound benzo(a)pyrene.

Complete = 1 ml incubation mixture containing 0.1 M phosphate buffer, pH 7.4, NADPH (1.2 μ mole) and protein (0.3 mg)

Incubation conditions	Oxidized products DPM x 10^2/10 min/mg microsomal protein with BP	with Cp-BP(B)
Complete	231	340
Complete + α-NF (0.1 mM)	21	76
Complete + BI (1 mM)	47	107
Complete + MP (5 mM)	60	137
Complete + SKF (1 mM)	76	196

α-NF, α-napthoflavone; BI, 1-benzylimidazole; MP, metyrapone; SKF, SKF-525A.

Table 4

Oxidation of protein (A) bound BP by microsomal monooxygenases

Complete = 1 ml incubation mixture containing 0.1 M phosphate buffer, pH 7.4, NADPH (1.2 μ mole) and microsomal protein (0.16 mg). BSA = bovine serum albumin.

Incubation conditions	Oxidized products DPM x 10^2/10 min/mg microsomal protein with BP	with Cp-BP(A)
Complete	349	363
Complete - NADPH	27	47
Complete + Microsomal Protein + Cp-BP(A)	-	57
Complete + boiled Cp-BP(A)	-	351
Complete + BSA (3.51 mg)	346	-

Aliquots of Cp-BP(A) taken contained 3.51 mg of protein.

emergence of GST activity along with the major fractions of second peak of protein bound BP radioactivity indicates that glutathione-S-transferases are possibly the major proteins for the binding of BP. In order to understand the biological functions of cytosolic protein binding of BP, both the pooled peak fractions of BP radioactivity were analyzed for their capacity to act as substrate for microsomal oxidation. Dependence of microsomal oxidation of protein (B) bound BP on protein concentration, incubation time and cofactors indicates that protein-bound BP can act as substrate for microsomal monooxygenases. The acceleration in the rate of microsomal oxidation of BP bound to cytosolic protein and its inducibility by 3-MC and inhibition by monooxygenase inhibitors suggest that protein-bound BP is a more effective substrate than ethanol suspended BP for microsomal oxidation. Since the major proteins of the second peak of protein-bound radioactivity contain high GST activity, it is intriguing to speculate that GST is at least one of the proteins that bind BP and facilitate its microsomal oxidation. Glutathione-S-transferases are a family of enzymes predominately present in cytosol which play a major role in cellular inactivation of a wide spectrum of electrophiles including carcinogens and mutagens; in rat liver they represent 5-10% of cytosolic protein (for references see review 15 and references therein). Of the several glutathione-S-transferases, GST-B (mol. wt. 44,000) has been shown to be identical with "ligandin", a major ligand binding protein in rat liver cytosol. In addition to GST, another binding protein is

present in liver cytosol at a concentration of approximately 10^{-4}M which binds selectively to amino azo dyes but has no catalytic activity (7). Since one of BP binding proteins in liver cytosol demonstrated catalytic activity of glutathione-S-transferase, it is likely that ligandin (GST-B) is involved in the binding of lipophilic carcinogens such as BP. Ligandin possesses a nonspecific hydrophobic binding site (7,15). Therefore it is assumed that BP binds to ligandin through the hydrophobic site and is transfered to the site of monooxygenase action without losing its substrate requirement for MFO action. It is conceivable that the presence of high concentrations of soluble proteins such as ligandin having hydrophobic binding sites and its capacity to bind many lipophilic carcinogens (7,15) including BP would facilitate the diffusion of lipophilic carcinogens to cross the cytosol from one lamella or one segment of endoplasmic reticulum to another. This would allow the microsomal monooxygenases, to better utilize lipophilic substrates of aryl hydrocarbon monooxygenases. It is prompting to hypothesize that GSTs including ligandin may serve as a carrier protein between one segment of ER and another in the transport of BP. Although the specificity and nature of BP binding to cytosolic proteins remains to be established, it is evident from this study that cytosolic protein binding of BP is a prerequisite for its intracellular transport from one segment of ER to another and for its microsomal oxidation.

ACKNOWLEDGMENTS

Supported in part by NIH Grant ES-1900 and NIOSH Grant OH-1149 and funds from Veterans Administration. Technical assistance of Warren Hubbard and J. Franklin Banks are gratefully acknowledged. Thanks are due to Mrs. Sandra Evans for typing.

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