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BENZO(A)PYRENE METABOLISM BY PURIFIED FORMS OF RABBIT LIVER MICROSOMAL CYTOCHROME P-450, CYTOCHROME b_5 AND EPOXIDE HYDRASE IN RECONSTITUTED PHOSPHOLIPID VESICLES

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SUMMARY

The roles of rabbit liver cytochrome b_5, epoxide hydrase and various forms of cytochrome P-450 in the NADPH-dependent metabolism of benzo(a)-pyrene were examined. After incorporation of the purified enzymes into phospholipid vesicles, using the cholate gel filtration technique, the various types of cytochrome P-450 did exhibit different stereospecificities in the oxygenation of the substrate. Cytochrome P-450_{LM2} was found to efficiently convert benzo(a)pyrene in the presence of epoxide hydrase to 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene whereas cytochrome P-450_{LM4} primarily participated in the formation of 9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene. By contrast, benzo(a)pyrene was not metabolized by cytochrome P-450_{LM3}. Cytochrome b_5 enhanced cytochrome P-450_{LM2}-catalyzed oxygenations 5-fold, whereas cytochrome P-450_{LM4}-dependent oxygenations proceeded at a 3 times higher rate when cytochrome b_5 was present in the membrane.

INTRODUCTION

Polycyclic aromatic hydrocarbons exert their carcinogenic effects only after metabolic activation to reactive metabolites that bind to critical cellular constituents (1-3). The occurrence of benzo(a)pyrene in our environment, and its potent carcinogenic activity have prompted numerous studies concerning the metabolism of this polycylic hydrocarbon and identification of its carcinogenic metabolites.

Benzo(a)pyrene is primarily metabolized in the cell by the combined action of the membrane-bound enzymes cytochrome P-450 and epoxide hydrase. As a result reactive intermediates are often formed thay may exert carcinogenic effects. Among different metabolic pathways that benzo(a)pyrene may undergo, the one leading to benzo(a)pyrene 7,8-dihydrodiol-9,10-oxide seems to be fatal with regard to cell transformation (4-6), whereas other benzo(a)-

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pyrene metabolites are more harmful to the cell (7). Since the monooxygenase system contains multiple forms of cytochrome P-450 with different specificities for a given substrate (8-10) the presence of one specific form of P-450 in a cell may thus determine the biological activity of e.g. benzo(a)pyrene. Therefore, it is of interest to examine the specificity of different purified forms of P-450 in the conversion of benzo(a)pyrene to reactive intermediates.

In previous papers we have characterized the properties of epoxide hydrase and the rabbit liver microsomal hydroxylase system in reconstituted phospholipid vesicles in comparison to the properties in a non-membranous system reconstituted with dilauroylphosphatidylcholine (10-13). We obtained altered catalytic properties of the enzymes upon incorporation into the vesicles as was evident from the substrate specificities of various types of cytochrome P-450, the reducibility of these proteins, their interactions with cytochrome b_5 (11)[2], and the kinetics of the reactions in the membrane-bound compared to the non-membranous system. In view of these findings it was considered of importance to evaluate the roles of various types of cytochrome P-450, cytochrome b_5 and epoxide hydrase in the metabolic transformation of benzo(a)-pyrene using this native type of reconstituted system.

MATERIALS AND METHODS

Materials. G-^{3}H Benzo(a)pyrene (specific activity 26 Ci per mmol) was obtained from the Radiochemical Centre, Amersham, England. Reference benzo(a)-pyrene metabolites were supplied by the ITT Research Institute, Chicago, Illinois, USA.

Assay and purification methods. Microsomal phospholipids were prepared according to Bligh and Dyer (15). Rabbit liver epoxide hydrase was prepared as described previously (12). The preparation had a specific activity of 434 nmol of styrene oxide hydrolyzed per mg of the protein and min and was homogeneous according to SDS-polyacrylamide gel electrophoresis. Electrophoretically homogeneous preparations of liver microsomal NADPH-cytochrome P-450 reductase, cytochrome P-450$_{LM2}$, P-450$_{LM3}$ and P-450$_{LM4}$ were prepared from phenobarbital-treated rabbits as previously described (13,14). The procedures are based on

[1]Abbreviations used: BP, benzo(a)pyrene; BP-9,10-diol, 9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene; BP-4,5-diol, 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene; BP-7,8-diol, 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene; P-450$_{LM}$, liver microsomal cytochrome P-450; P-450$_{LM2}$, P-450$_{LM3}$ and P-450$_{LM4}$, forms of P-450$_{LM}$ designated according to their electrophoretic properties; SDS, sodium dodecyl sulphate;

those presented by Yasukochi and Masters (16) and Haugen and Coon (17), respectively. The specific contents of the proteins were: P-450$_{LM2}$, 12.5-14.2 nmol/mg; P-450$_{LM3}$, 9.5 nmol/mg; P-450$_{LM4}$, 10.5 nmol/mg; NADPH-cytochrome P-450 reductase 12.5 nmol of flavin/mg when flavin was detected by the absorption at 456 nm of the protein, using the absorption coefficient 10.7 $mM^{-1}cm^{-1}$ (18). Cytochrome b_5 was purified as described elsewhere[2] to a specific content of 28.2-34.5 nmol/mg and the preparations were electrophoretically homogeneous. Cytochrome P-450 was measured according to Omura and Sato (19) using 91 mM^{-1} cm^{-1} as absorption coefficient. Cytochrome b_5 was detected using the absorption coefficient 100 $mM^{-1}cm^{-1}$ for the absorbance difference between the reduced minus the oxidized form of the protein at 424 nm (20). Protein was determined according to Lowry (21).

Preparation of vesicles. Unilamellar phospholipid vesicles containing the various proteins were prepared from microsomal phospholipids using the cholate gel filtration technique previously described (12,14,22). The vesicles were usually prepared devoid of cytochrome b_5. The subsequent addition of cytochrome b_5 to the vesicles at 37°C resulted in a rapid and complete incorporation of the protein into the vesicles as judged from results obtained by Sepharose 4B chromatography of the preparations.

Analysis of benzo(a)pyrene (BP) metabolism. Incubations with benzo(a)pyrene were performed using vesicles corresponding to 0.2 μM of P-450 having a molar ratio of cytochrome P-450:NADPH-cytochrome P-450 reductase:epoxide hydrase:phospholipid of 3:1:3:100 in 1 ml 50 mM potassium phosphate buffer, pH 7.4. Benzo(a)pyrene (25 nmol) with 500 000 dpm ^{3}H-BP in 40 μl of methanol was added and after 3 min preincubation at 37°C, the reactions were started by the addition of 0.5 mg NADPH in 100 μl of water. Control incubations were performed in the absence of NADPH. The incubations were terminated after 10, 20 or 30 min by the addition of 1 ml of acetone. The incubation mixtures were extracted with 2 x 2 ml of ethyl acetate, containing 0.08% (w/v) of butylated hydroxytoluene. The combined organic layers were dried with 0.5 g of anhydrous sodium sulphate and the ethyl acetate was removed under a stream of nitrogen. The incubations were stored in the dark under nitrogen at -20°C until HPLC analysis. Separation of the different benzo(a)pyrene metabolites was performed on an LDC High Pressure Liquid Chromatograph equipped with two Constametric pumps and a Bondapack C_{18} column (1.52 x 25 cm). The samples were dissolved in 50 μl of acetone and 20 μl aliquots were injected onto the column. The metabolites were eluted with a linear 50 min gradient of elutent (2.2 ml/min) from 60% methanol in water to 100% methanol. Fractions were collected each 40 sec directly into counting vials for an Intertechnique SL-30 liquid scintillation spectrometer. Counting was performed using 3 ml of Lumagel^R as scintillator liquid.

RESULTS

Benzo(a)pyrene Metabolism in the Reconstituted Vesicles

Two major phenolic fractions were isolated from the incubation mixtures containing the reconstituted vesicles and were eluting together with authentic 9- and 3-hydroxybenzo(a)pyrene from the HPLC-column. In addition benzo(a)pyrene quinones and three different benzo(a)pyrene dihydrodiols, i.e. BP-9,10-diol, BP-4,5-diol and BP-7,8-diol, were detected. The formation of the metabolites by the reconstituted vesicles was linear for 20-30 min (Fig. 1).

[2] Ingelman-Sundberg, M., and Johansson, I., submitted for publication.

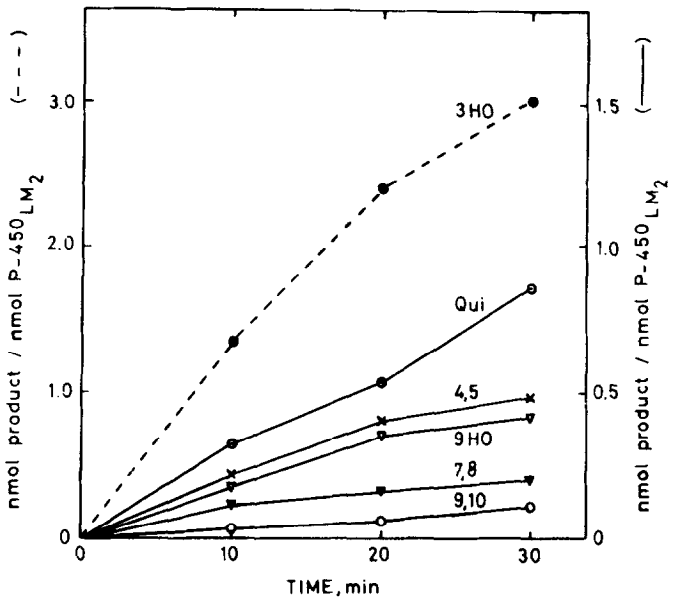

Fig. 1. Time courses of cytochrome P-450$_{LM2}$-dependent oxygenations of benzo(a)pyrene in reconstituted phospholipid vesicles. The vesicles were prepared by the cholate gel filtration technique from microsomal phospholipids, cytochrome P-450$_{LM2}$, NADPH-cytochrome P-450 reductase, cytochrome b_5 and epoxide hydrase in a molar ratio of 1000:2:1:0.8:3. Incubations were performed as described under "Methods". Further explanations in the text.

The metabolism of benzo(a)pyrene by phospholipid vesicles containing various forms of rabbit liver microsomal cytochrome P-450 is summarized in Table I. Cytochrome P-450$_{LM_2}$ was the most active form of the cytochromes P-450 in transforming benzo(a)pyrene into more polar products. Inclusion of epoxide hydrase in the vesicles resulted in the formation of various dihydrodiols; the major metabolite isolated from incubation mixtures containing P-450$_{LM_2}$ was the 4,5-diol, comprising about 15% of the metabolites, whereas much smaller amounts of BP-9,10-diol and BP-7,8-diol were formed.

Cytochrome P-450$_{LM_3}$, a non-inducible form of cytochrome P-450, was markedly inactive in metabolizing benzo(a)pyrene in the phospholipid vesicles. Only very small amounts of phenol II (eluting together with authentic 3-hydroxybenzo(a)pyrene from the HPLC column) was isolated from P-450$_{LM_3}$-containing incubation mixtures.

The regiospecificity of cytochrome P-450$_{LM_4}$ against benzo(a)pyrene was different compared to cytochrome P-450$_{LM_2}$. Although the overall activity was

Table I. Metabolism of benzo(a)pyrene in reconstituted phospholipid vesicles containing NADPH-cytochrome P-450 reductase, different forms of cytochrome P-450$_{LM}$ and, if indicated, epoxide hydrase (EPH) and cytochrome b_5. Incubations were performed as described under "Methods" using vesicles corresponding to 0.2 nmol P-450. The molar ratio of P-450:P-450-reductase:cytochrome b_5:epoxide hydrase (EPH):phospholipid used was 3:1:2:3:1000. The values represent means from 2-6 experiments and were calculated from the time curves obtained from incubations performed for 10, 20 and 30 min.

	Catalytic activity, pmol of product/nmol of P-450, min.						
Composition of vesicles	BP 9,10-dihydrodiol	BP 4,5-dihydrodiol	BP 7,8-dihydrodiol	BP Quinones	BP phenols I (9 HO)	BP phenols II (3 HO)	TOTAL
LM_2	a	1.5	a	19	20	90	130.5
LM_2 + b_5	a	0.8	a	87	56	196	339
LM_2 + EPH	1.1	13.4	2	15.8	8.2	48	87.2
LM_2 + b_5 + EPH	6.1	38	14	76	45	255	434.1
LM_3	a	a	a	a	a	2	2
LM_3 + EPH + b_5	a	a	a	a	a	20	20
LM_4	1	a	a	3.5	3.5	14.4	22.4
LM_4 + EPH	5.5	2.3	1.1	3.2	2.3	8.5	22.9
LM_4 + b_5 + EPH	13	4.1	3.7	8.5	5.8	35	70.1

[a] = less than 0.5 pmol of product was formed per nmol P-450, min.

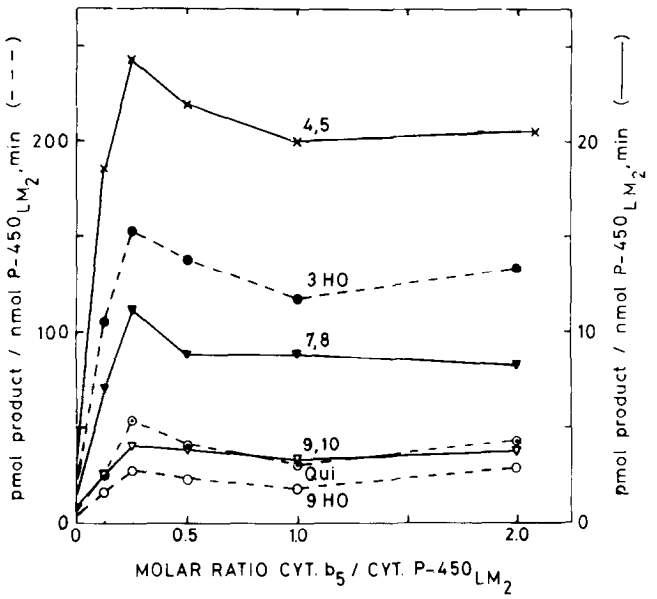

Fig. 2. Cytochrome b_5-dependent stimulation of cytochrome P-450_{LM2}-catalyzed oxygenations of benzo(a)pyrene in reconstituted phospholipid vesicles. The vesicles were prepared from microsomal phospholipids, cytochrome P-450_{LM2} and NADPH-cytochrome P-450 reductase in a molar ratio of 1000:4:1 and indicated amounts of cytochrome b_5 were subsequently added to the vesicles at 37°C. After 3 min. incubations with benzo(a)pyrene were performed as described under "Methods".

only about 25% of that exhibited by P-450_{LM_2}, six times higher amounts of BP-9,10-diol were produced by phospholipid vesicles containing P-450_{LM_4}. In addition lesser amounts of BP-4,5-diol and BP-7,8-diol were isolated when epoxide hydrase was present. In contrast to the situation when using P-450_{LM_2}, as much as 40% of the benzo(a)pyrene metabolites isolated comprised of dihydrodiols, more than 60% being attributed to BP-9,10-diol.

Effects of cytochrome b_5. As seen from Table I the introduction of nearly equimolar amounts of cytochrome b_5 enhanced the cytochrome P-450-dependent metabolism of benzo(a)pyrene 5-fold in case of cytochrome P-450_{LM_2}-dependent activities and 3 times when cytochrome P-450_{LM_4}-catalyzed reactions were examined. When vesicles containing P-450_{LM_2}, NADPH-cytochrome P-450 reductase and epoxide hydrase were titrated with cytochrome b_5 (Fig. 2) concomitant stimulations were seen in all P-450_{LM_2}-dependent activities towards benzo(a)pyrene as the amount of cytochrome b_5 was increased in the vesicles. These

results, in addition to those presented in Table I, indicate no change of the stereospecificity in the oxygenation reactions when cytochrome b_5 is present in the vesicles. Maximal stimulation by cytochrome b_5 was obtained at a 1:1 molar ratio of cytochrome b_5:NADPH-cytochrome P-450 in the vesicles, i.e. at a ratio of b_5 to P-450_{LM_2} of 1:0.25. This is in accordance with previous results[2], indicating the formation of a functionally active ternary complex between the proteins in the vesicles.

DISCUSSION

The results presented indicate that the various types of cytochrome P-450 have markedly different stereospecificities in the metabolism of benzo-(a)pyrene. The phenobarbital-inducible form of cytochrome P-450, P-450_{LM_2}, primarily participates in the formation of the non-toxic BP-4,5-diol whereas the 3-methylcholantrene inducible P-450-form, P-450_{LM_4}, preferentially catalyzes the 9,10-epoxidation of the substrate and may thus participate in the formation of the ultimate carcinogen of BP. Cytochrome P-450_{LM_3}, an uninducible P-450-form, did not metabolize benzo(a)pyrene. Previous investigations of benzo(a)pyrene metabolism by purified rabbit liver microsomal P-450:s (23,24) have been hampered by absence of epoxide hydratase in the system, thereby not making the detection of the primary benzo(a)pyrene metabolites possible.

By using phospholipid vesicles for reconstitution of benzo(a)pyrene metabolism it was possible to obtain a 3-5-fold stimulation by cytochrome b_5 in the oxygenation reactions without any significant changes in the regiospecificities in the reactions. Obviously, the previously raised explanation for the action of cytochrome b_5 in the NADPH-supported cytochrome P-450-catalyzed oxygenations in donating the second electron to P-450 more efficiently than NADPH-cytochrome P-450 reductase, thereby enhancing the overall hydroxylation rate[2] in the vesicles, seems plausible also in case of benzo(a)-pyrene as substrate.

In conclusion, the results here presented indicate the regiospecificity of benzo(a)pyrene metabolism being preferentially associated with the type of cytochrome P-450 present in the vesicles, whereas cytochrome b_5 and epoxide are of secondary importance in this respect. The interindividual variations in humans in aryl hydrocarbon hydroxylase activities (25,26) and, furthermore, in susceptibility to carcinogenic action of e.g. cigarette smoke, may well be attributed to variations in the composition of the cytochrome P-450 enzymes in a given tissue.

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