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INDUCTION OF BENZO(a)PYRENE HYDROXYLASE IN ASPERGILLUS OCHRACEUS TS: EVIDENCES OF MULTIPLE FORMS OF CYTOCHROME P-450

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SUMMARY : The filamentous fungus Aspergillus ochraceus TS produces an inducible microsomal cytochrome P-450 linked monooxygenase which is capable of hydroxylating benzo(a)pyrene in presence of O_2 and NADPH. The addition of Benzo(a)pyrene, 3-Methyl cholanthrene, β-Napthoflavone and other aryl hydrocarbons during the induction period causes drammatic improvement in the kinetics of benzo(a) pyrene hydroxylation as was evidenced by large decrease in K_m and increase in V_{max} values. On the other hand, treatment with Phenobarbital, Polychlorinated biphenyl and Progesterone has no significant effect on the kinetics of benzo(a)pyrene hydroxylation although a significant induction of NADPH-Cyt C reductase activity was observed in all the three cases. Again, both Phenobarbital and 3-Methyl cholanthrene induced microsomes exhibit the characteristic reduced metyrapone difference spectra. These findings together with the results obtained with flavone on the metabolism of benzo(a) pyrene by various microsomal preparations suggest a parallel induction of multiple forms of cytochrome P-450 as observed in mammalian liver under identical condition.

INTRODUCTION : The filamentous fungus Aspergillus ochraceus TS was found to possess an inducible microsomal cytochrome P-450 (Cyt P-450) containing monooxygenase system, catalyzing the hydroxylation of steroids and many aryl hydrocarbons especially benzo(a) pyrene (1,2) very efficiently with many properties similar to that found in mammalian liver. We have described earlier (2) the isolation of a highly reactive microsomes, catalyzing the oxidation of benzo(a)pyrene (BP) and the influence of different classical inducers and modifiers of mammalian Cyt P-450 on this hydroxylation.

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The present paper describes the influence of different inducers of Cyt P-450 on the kinetic parameters of BP hydroxylation (i.e. K_m and V_{max}) and also on different components of the hydroxylase system. We also provide data on the effect of flavone on the metabolism of BP by microsomes prepared after pretreatment with different inducers; thus suggesting the possible existence of multiple forms of Cyt P-450 in this fungus. It is suggested that activation or inhibition of BP metabolism by flavone depends on the form or types of Cyt P-450 used in the study (3). The use of different inducers and specific compounds to manipulate the biochemical and biophysical properties of the microsomal hydroxylation system has played a major role in establishing the existence of multiple forms of cytochrome P-450.

MATERIALS AND METHODS : Benzo(a)pyrene, 3-methyl cholanthrene (3-MC), β-Napthoflavone (BNF), phenobarbital (PB), naphthalene, phenanthrene, progesterone, NADPH (Type IX) and flavone were purchased from Sigma (U.S.A.). Polychlorinated biphenyl (PCB, Aroclor 1254) was kindly provided as a gift from Prof.G.C.Chatterjee, Dept. of Biochemistry, Calcutta University. 3-hydroxy benzo(a)pyrene was also received as a gift from The National Cancer Institute, Chemical Repository at the IIT Research Institute, Chicago,Illinois. All other chemicals used were purchased from local sources and were of analytical grade.

Cultivation and Induction : Growth and induction of A.ochraceus TS used in this investigation was done in liquid medium by the method described previously (1,2). Cells were grown in a liquid medium (composition %,w/v : sucrose 1.0, cornsteep liquor 0.5 and K_2HPO_4 0.05, pH 6.5 before sterilization) for 48 h after which inducing agents were added followed by further incubation for 16-18 h (induction period).

Preparation of microsomes : Microsomes were prepared by differential centrifugation after the disruption of washed mycelium of A.ochraceus TS in 0.1M phosphate buffer pH 7.6 containing 0.01M EDTA, 0.01M GSH and 0.25 M sucrose (buffer A) by the methods described earlier (1,2). The microsomal fraction (105,000 g pellet) was washed and resuspended in buffer A containing 0.01M KCl by the use of hand held potter type homogenizer.

Enzyme assays : BP hydroxylase activity was measured as in (2) by detecting the amount of 3-hydroxy benzo(a)pyrene produced by fluorimetric method of Nebert and Gelboin (4). Unless otherwise stated, the typical incubation mixture contained 0.15 μ moles BP, 0.5 μ moles NADPH and microsomal protein (0.6-1.0 mg) in 0.1M phosphate buffer pH 7.6 in a total volume of 1 ml. In experiments with flavone, microsomes were preincubated with its various concentrations in 40 μl of acetone before the addition of substrate and NADPH. The reaction mixtures were incubated at 28°C on a rotary

shaker for 15 min and fluorescent phenolic metabolites were measured as previously described (2) in a Perkin-Elmer Spectrofluorometer (MPF 44B). NADPH-Cytochrome C reductase activity was determined by the method described by Williams and Kamin (5). Metyrapone difference-dithionite reduced Cyt P-450 spectra in the microsomes were recorded following the same procedure (6). Metyrapone dissolved in water were added to a concentration of 200 µm. Protein was estimated according to the method of Lowry et al. (7) using bovine serum albumin as standard.

RESULTS AND DISCUSSION : The filamentous fungus A.ochraceus TS contains a microsomal BP hydroxylase which has been characterised as a Cyt P-450 linked monooxygenase (2). The hydroxylase activity could be induced by BP to an activity sevenfold that of non-induced cells (2). Addition of BP at different concentrations (40-160 µm) during the induction period had a dramatic effect on the kinetics of BP hydroxylase activity when measured in microsomal pellet by means of double reciprocal Lineweaver-Burk plot. It has been found that with increasing concentrations of BP in the medium there is a lowering of Michaelis constant (K_m) i.e. increasing affinity for BP and elevation of maximal velocity (V_{max}). BP is well-known as an inducer of BP hydroxylase in mammals, where it has exhibited similar effects on the kinetics of this enzyme (8.9). In the present study the alteration of kinetic parameters after BP treatment indicates the induction of a particular form of Cyt P-450 which has high affinity and turnover for BP hydroxylation and thus suggesting its similarity with Cyt P-448 which is induced in rat liver by 3-MC treatment. It is a well-known fact that microbial monooxygenases of Cyt P-450 type are inducible by substrates. In a limited number of cases classical mammalian Cyt P-450 inducers have been examined and some parallels to the induction of monooxygenase in mammals have been observed (10). Table 1 shows the effect of different inducing agents on the kinetic parameters of BP hydroxylase activity as well as on the NADPH-Cyt C reductase activity. Pretreatment with 3-MC, BNF and other aryl hydrocarbons, used as inducers, results

Table I. Effect of inducing agents on NADPH-Cyt C reductase and kinetics of BP hydroxylase system in A.ochraceus TS.

Inducer added		BP hydroxylase K_m (μm)	BP hydroxylase V_{max} (nmoles hydroxy BP/min/mg protein	NADPH-Cyt C reductase (nmoles/min/ mg protein)
0		250	0.11	30.25
Benzo(a)pyrene (BP)	40 μM	70	0.37	45.35
	80 μM	50	0.60	45.75
	120 μM	45	0.64	46.00
	160 μM	45	0.69	46.00
3-Methyl Cholanthrene (3-MC)	80 μM	60	0.90	42.00
	100 μM	45	1.00	42.30
	120 μM	45	1.10	45.00
β-Naphthoflavone (BNF)	100 μM	44	0.80	59.00
Phenanthrene	100 μM	60	1.00	49.00
Naphthalene	100 μM	66	0.90	48.00
Phenobarbital (PB)	100 μM	133	0.39	150.00
Polychlorinated biphenyl (PCB, Aroclor 1254)	100 μM	125	0.32	115.00
Progesterone	100 μM	150	0.39	78.00

in a significant improvement of K_m and V_{max} for BP hydroxylation and may be considered to be a selective induction of a form (or forms) of Cyt P-450 with a high activity towards BP hydroxylation. This is substantiated by the fact that these agents do not increase the levels of NADPH-Cyt C reductase a key enzyme of the P-450 containing monooxygenase, thus resembling the general characteristic of inducers of relatively narrow specificity Cyt P-448 (10). On the other hand, PB, PCB and progesterone induce the BP hydroxylase to some extent as was evidenced by slight modification of K_m and V_{max}; although a significant induction of NADPH-Cyt C reductase (2.5 to 5 fold) is observed which is a common characteristic of inducer of broad specificity Cyt P-450 in mammals (10). The induction of BP-hydroxylase activity after pretreatment with BNF is

highly consistent with previous results (11) where it has been reported to cause a selective synthesis of a form of Cyt P-450 with a high affinity and turnover for BP. Induction with 3-MC and other aryl hydrocarbons shows striking resemblance with hepatic microsomes and hamster fetus cells (12.13). In all cases, a large decrease in K_m and elevation of V_{max} was observed suggesting the induction of enzyme with higher affinity and turnover for BP. In the present study, PCB was also tried as an inducer as this relatively highly chlorinated mixture of compounds (54% chlorinated) is reported to be an effective inducer of all isozymes produced in rabbit liver after treatment with PB and 3-MC (14). The results reported here suggests its similarity with principal Cyt P-450 obtained from the liver of PB induced rabbit. Pretreatment with PCB causes only slight improvement of K_m and V_{max}. Again, both PB and 3-MC induced microsomes exhibited the characteristic metyrapone-dithionite reduced spectra (Fig.1) and the results are highly consistent with those obtained with purified Cyt P-450 from PB and 3-MC treated rats (6). Only the PB induced microsomes produces a significant absorption (at 446-447 nm) while the other does not (Fig.1). So from the above studies it may be concluded that different classi-

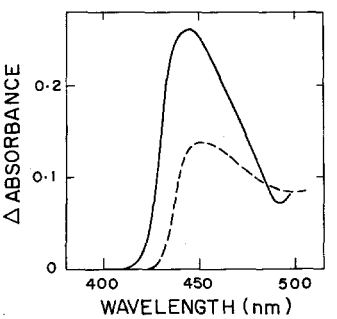

Fig.1 : Difference spectra of reduced microsomes from PB (———) and 3-MC (------) treated A.ochraceus TS with Metyrapone. The microsomes were suspended in 0.05M phosphate buffer (pH 7.5) containing 15mM KCl and 20% glycerol to a final concentration of 2 mg/ml and divided into two 3 ml cuvettes. The spectra were recorded in Cary Model 17D spectrophotometer.

cal inducers of mammalian Cyt P-450 can induce the BP hydroxylase activity to varying extent and thus suggesting the existence of multiple forms of Cyt P-450 in this fungus. Although, attempts to show the differences in the reduced CO-difference spectra, which has played a major role in establishing the existence of multiple forms of Cyt P-450, were proved to be unsatisfactory due to the inherent presence of cytochrome oxidase in the microsomal preparation which is shown to obliterate the Co-difference spectra by shifting the absorption maxima at longer wavelength (452-454 nm). Moreover, it was not even possible to show the characteristic CO-difference spectra when measured in microsomal pellet after PB, PCB and progesterone treatment, according to the method of Omura and Sato (15); as there were always a negative intense peak at 442-444 nm with a maximum at 427-430 nm (Ghosh,D.K. and Samanta, T.B., unpublished observation). On the other hand, some compounds were found to react selectively with various forms of microsomal Cyt P-450 and useful informations could be derived by studying these selective interactions. In the present study, flavone is found to affect differentially the BP metabolism in the microsomes prepared after pretreatment with different inducers and has been used to probe the relative distribution of different forms of Cyt P-450 in various microsomal preparations. It has been suggested that the specificity in the effects of flavone on the metabolism of BP resides on the type of Cyt P-450 used under investigation (3). Fig.2 shows the effect of flavone on the metabolism of BP by various microsomal preparations. It has been found that both 3-MC and BP induced microsomal metabolism were inhibited by flavone indicating its similarity with those obtained in rat liver microsomes after 3-MC treatment (16) and also with Cyt $P\text{-}450_{LM_6}$ which is produced in rabbit liver after induction with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (3). On the other hand, the addition

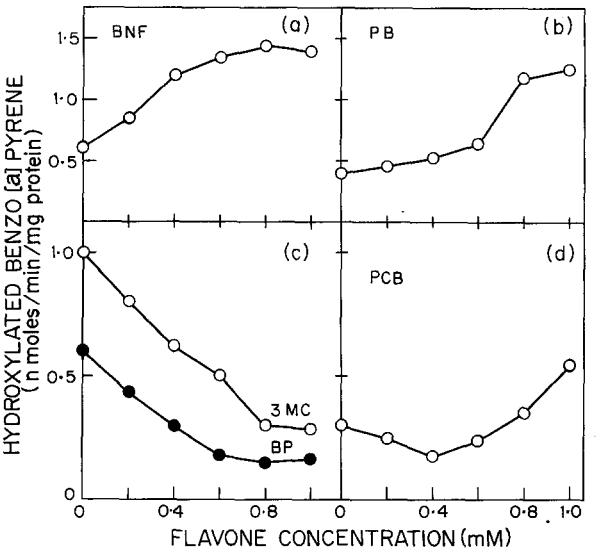

Fig.2 : Effect of flavone on NADPH supported BP hydroxylation by microsomes prepared after pretreatment of A.ochraceus TS with (a) BNF (b) PB (c) 3-MC and BP (d) PCB. Hydroxylation was measured as described under Materials and Methods.

of flavone (0.2-1.0 mM) to the PB and BNF induced microsomes stimulated the BP metabolism by 2 to 3 fold and thereby indicating its similarity with Cyt P-450$_{LM_4}$ which is the major form produced in rabbit liver after treatment with BNF and TCDD (3). But the result obtained with PCB induced microsomes is really puzzling as the BP metabolism was found to be inhibited at low and activated at high concentration respectively. As PCB induction mimicks that observed by combined administration of PB and 3-MC (14) there may be possibility of synthesis of different forms of Cyt P-450 to varying extent; one of these forms (minor form and possibly similar to Cyt P-450$_{LM_6}$) is first acted upon by flavone resulting in inhibition while the other (major form and possibly similar to Cyt P-450$_{LM_4}$) is subsequently interacted by flavone causing stimulation of benzo(a) pyrene hydroxylation. Further studies are needed to elucicidate the actual mechanism. The quantitative analysis of the metabolite profiles by various microsomal preparations has not yet been made. Preliminary results revealed the appearence of various quinones and phenolic

metabolites of BP. A study of BP metabolites generated by each microsomal preparation is now underway using high performance liquid chromatography. Attempts are also being made to develop a standarized SDS-gel electrophoresis procedure so that Cyt P-450 monooxygenases after various induction treatments can be compared and this also help in comparative induction studies with mammalian and microbial microsomal preparations.

The results demonstrate not only the existence of multiple forms of Cyt P-450 in A.ochraceus TS but also provide evidences in suggesting a biochemical relatedness in the metabolism of BP with liver microsome (17).

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