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MICROSOMAL BENZO(a)PYRENE HYDROXYLASE IN ASPERGILLUS OCHRACEUS TS: ASSAY AND CHARACTERIZATION OF THE ENZYME SYSTEM

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SUMMARY : Microsomal preparations of Aspergillus ochraceus TS oxidised benzo(a)pyrene very efficiently in the presence of NADPH and O_2 and exhibits a pH optimum of 8.0-8.2. The hydroxylation is also effected in presence of $NaIO_4$. Hydroxylation was inhibited by metyrapone, SKF-525A, PCMB, imidazole, carbon monoxide and flavone but not by cyanide, azide and antimycin A indicating thereby the involvement of cytochrome P-450 in this reaction. Inhibition by cytochrome C is consistant with the participation of NADPH-cytochrome C reductase in this hydroxylation. Reduced microsomes and its solubilized preparation, when treated with carbon monoxide, showed absorption maxima at 453 and 449 respectively. Different classical inducers of cytochrome P-450 induce the benzo(a)-pyrene hydroxylase activity to varying degree and as such suggests the existence of multiple forms of cytochrome P-450 in this fungus.

INTRODUCTION : Interest in the metabolism of polycyclic aromatic hydrocarbon benzo(a)pyrene by higher organism stems from the fact that it is a wide spread environmental pollutant that possesses toxic, mutagenic and carcinogenic activities. The mammalian liver possesses a cytochrome P-450 containing monooxygenase which incorporates oxygen into this compound to produce more water soluble one so that it can be readily excreted from the body. In contrast studies on the metabolism of such compound by fungi are limited (1,2). However, the results suggest a biochemical relatedness in the metabolism of benzo(a)pyrene by fungal and liver microsomes (3). In particular Cunninghamella bainieri catalyzes the oxidation of aromatic compounds in the same way as hepatic microsomes as the benzo(a)pyrene hydroxylase activity in the former was also found to be associated with a cyt P-450 containing monooxygenase located in the microsome.

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The present paper reports the condition for ideal metabolism of benzo(a)pyrene in a filamentous fungi Aspergillus ochraceus TS by cell free extract, location and resolution of the enzyme system, involvement of cytochrome P-450 and effect of different classical modifiers and inducers of mammalian Cyt P-450 on this hydroxylation. To our knowledge this seems to be a first report on benzo(a)pyrene (BP) metabolism in a strain belonging to the genus Aspergillus.

MATERIALS AND METHODS : Aspergillus ochraceus TS used in this investigation was grown in liquid medium by the method previously described (4,5). Growth was for 48 h on a rotary shaker at 28-30° in a medium containing 1% sucrose, 0.5% cornsteep liquor and 0.05% K_2HPO_4, followed by the addition of inducer benzo(a)pyrene (BP, 80 μM) and further incubation for 16-18 h (induction period). Induction by other substances were performed exactly in the same way. Preparation of cell free extract : After the induction period the mycelium was filtered and washed successively with 0.5% NaCl solution and then with 0.1 M phosphate buffer pH 7.6. It was then pressed between filter paper and kept in deep freeze (-20°) for 2 h. The cell free extract was prepared in 0.1 M phosphate buffer pH 7.6 containing 0.01 M EDTA, 0.01 M GSH and 0.25 M sucrose (Buffer A) at 4° exactly in the same way as described before (5). Sub-cellular fractions were prepared by differential centrifugation of cell free extract at 18,000 and 105,000 g respectively. The microsomal fraction (105,000 g pellet) was washed and resuspended in buffer A containing 0.01 M KCl by the use of hand held potter type homogenizer. Assay and Incubation condition : The benzo(a)pyrene hydroxylase activity was measured by detecting the amount of 3-hydroxy benzo(a)-pyrene produced by the fluorimetric method of Nebert and Gelboin (6). The typical incubation mixture contained 0.1 umoles BP dissolved in dimethyl sulfoxide, 0.5 umoles NADPH and microsomal protein (0.6-1.0 mg) and 0.1 M phosphate buffer pH 7.6 in a total volume of 1 ml. The hydroxylation was initiated by the addition of NADPH and the incubation was carried out at 28° on a rotary shaker for 15-30 min as the situation demanded. The reaction was terminated by the addition of 3 ml of a mixture of hexane:acetone (2:1) and shaken for 5 min and centrifuged. The organic layer was then extracted with 3 ml of 1 N NaOH. The alkali extract was read at 522 nm in a Perkin-Elmer spectrofluorometer (MPF 44B) with excitation at 396 nm. The concentration of hydroxy BP was determined from a standard curve derived from an authentic sample of 3-hydroxy BP, kindly provided by The National Cancer Institute, Chemical Repository at the IIT Research Institute, Chicago, Illinois. In routine experiments quinine-HBr in 0.1 N H_2SO_4 was used for calibration of the spectrofluorometer. In all experiments all the tubes were covered with black paper. Carbon monoxide difference spectrum was recorded by the method of Omura and Sato (7). Microsome was solubilized by resuspending washed microsomal protein at a concentration of 10 mg/ml in 15 ml of 0.1 M phosphate buffer pH 7.6 containing 0.01 M EDTA, 0.01 M GSH, 0.25 M sucrose and 1.2% sodium cholate and was immediately stirred at 5°C for 30 min. The solution was then centrifuged at 105,000 g for 1 h at 4°C. The supernatant thus obtained was then dialysed against 0.01 M phosphate buffer pH 7.6 containing 0.01 M EDTA, 0.01 M GSH, 0.25 M sucrose and 0.1% sodium cholate for 6 h at 4°C. Co-difference spectra was then recorded in dialysate as usual. The NADPH cytochrome C reductase activity was measured at 550 nm in the described method (8). Protein was estima-

ted according to the method of Lowry et al. (9) using bovine serum albumin as standard.

RESULTS AND DISCUSSION : In the present work cell free extract (CFE) from BP induced cells of A.ochraceus TS was prepared following certain precise conditions different from that used in the case of C.bainieri (2). It has been found that the presence of EDTA and GSH in homogenization buffer is essential for preparation of cell free extract with high activity. Again, the homogenization carried out at pH 7.1 or below irrespective of buffer used, yielded enzyme preparation with low hydroxylase activity (15-20% of the optimal activity). On the other hand, there is no subtle difference in activity by either using 0.1 M phosphate buffer or 0.1 M Tris HCl (both having pH 7.6) or using buffer of pH higher than 7.6 for homogenization. It is quite possible that presence of EDTA and a pH of 7.6 of the medium could inhibit the proteolytic activity of the cell free extract, thus preventing the inactivation of hydroxylase system. Again to have a good hydroxylase activity A.ochraceus TS requires definite induction time (16-18 h in our case) and cells should be harvested from exponential growth phase (54-62 h). Cells harvested at early log phase or near stationary phase showed poor activity. On the other hand, the microsome obtained from non-induced cells of A.ochraceus TS shows low level of activity as compared with those prepared from induced cells (Table 1). Again, there was no significant increase in BP hydroxylation by the microsomes prepared from cells grown in presence of different concentration of glucose although the need for presence of fermentable sugars for the synthesis of Cyt P-450 is indicated (10).

Differential centrifugation studies indicate that most of the BP hydroxylase activity was associated with microsomes which also contain highest NADPH Cyt C reductase activity (Table 1). A good amount of activity was also found in post mitochondrial supernatant. Either post microsomal supernatant (PMS) or mitochondrial pellet contained very little activity and addition of PMS to the microsomes did not enhance the hydroxylase activity. In the case of C.bainieri (2) most of the hydroxylase activity (about 95%) was concentrated in microsomes whereas PMS contained most of the reductase activity (90%).

The BP hydroxylation was linear upto 30 min as revealed by its kinetic studies (Fig.1a) after which it did not increase significantly which is in contrast to that obtained in C.bainieri where

Table I. Activities of different components of BP hydroxylase system of A.ochraceus TS in different sub-cellular fraction and in the solubilized preparation of microsomes.

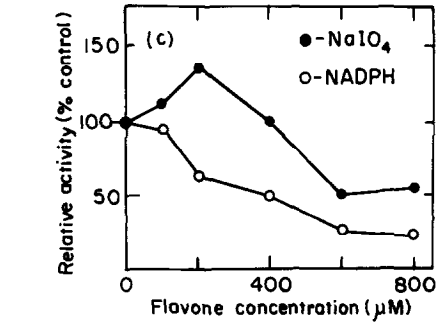
Fraction	BP hydroxylase activity (nmoles/min/mg)	NADPH-Cyt C reductase activity (nmoles/min/mg)
1500 g supernate (CFE)	0.18	nd
18,000 g pellet (Mitochondria)	0.06	nd
18,000 g supernate (Post mitochondrial supernate)	0.45	40.00
105,000 g pellet (Microsome)	0.60	45.75
105,000 g supernate (PMS)	0.08	6.25
Solubilized Microsomes	0.79	75.75
Microsomes (from non-induced mycelia)	0.09	30.25

nd - not determined.

a long assay time of 2 h was chosen due to the presence of an inhibition period during first 90 min. The sp.activity was found to be 0.6 nmoles/min/mg protein which was very high in contrast to previous results (2) and so have been compared with those obtained by hepatic microsomes and hamster fibroblast (6,11).

The pH optimum of the hydroxylase was 8.0-8.2 (Fig.1b). However, a pH of 7.6 was used in all cases for comparison of all data as the activity at pH 7.6 is 80% of the optimal activity.

The hydroxylase activity of A.ochraceus TS was dependent on NADPH and O_2. NADH was less effective. Ascorbate or tetrahydrocholate can not serve as cofactors. Activity was stimulated by using NADPH regenerating system. No synergistic effect was observed when NADH was added to NADPH. Again, oxidising agents like $NaIO_4$ and $NaClO_2$ (optimum concentration being 5-6 mM) can support this hydroxylation and as such represent the involvement of Cyt P-450 in this hydroxylation. Specific activity in $NaIO_4$ supported hydroxylation was calculated to be 0.47 nmoles/min/mg. In fact, these hydroxylating agents bypass the reduction step of NADPH dependent pathway and form the ferryl ions $[Fe^{+4}]$ (12).



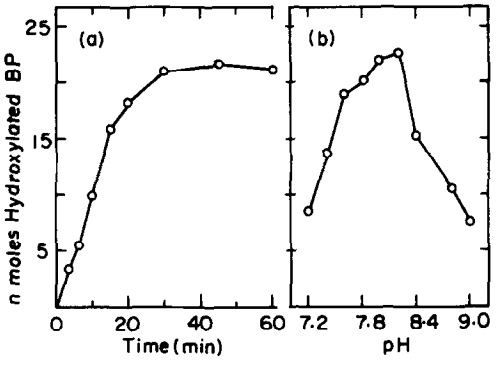


Fig. 1a : Kinetics of NADPH supported microsomal BP hydroxylation.

Fig. 1b : Effect of pH on microsomal BP hydroxylase activity with the use of Tris-HCl buffer. Assays are conducted as described in the methods.

Fig. 1c : Effect of flavone on NADPH and NaIO4 dependent BP hydroxylation by microsome from BP-induced A.ochraceus TS. The activity of the controls in presence of NADPH and NaIO4 were 0.6 and 0.47 nmoles/min/mg protein respectively.

As shown in Table II the microsomal hydroxylase activity was inhibited to a significant extent by Co, metyrapone, SKF-525A, imidazole, PCMB but not by CN^-, N_3^- or antimycin A, typical inhibitors of mitochondrial electron transport chains, indicating the involvement of Cyt P-450. The inhibitory effect of Cyt C is consistent with the participation of a Cyt C reductase in this hydroxylation. Again, the participation of Cyt P-450 in this hydroxylation was substantiated by the fact that both the microsomes and its solubilized preparation from BP induced A.ochraceus TS showed the characteristic peak of Cyt P-450 at 453 and 449 nm respectively. The shift in the absorption maxima at longer wavelength (453 nm) by microsomal pellet is due to the possible contamination with cytochrome oxidase, the existence of which was further evidenced by the presence of a peak at 428 (absorption maxima of Cyt oxidase was

Table II. Effect of modifiers on microsomal BP hydroxylase activity.

Modifier	Relative enzyme activity*
Control	100
Metyrapone (2 mM)	40
SKF-525A (2 mM)	10
Imidazole (2 mM)	50
Azide (2 mM)	100
KCN (2 mM)	100
Antimycin A (2 mM)	100
Cyt C (0.01 mM)	50
(0.1 mM)	20
CO (Bubbled for 2-3 min)	20
PCMB (1 mM)	10
Co^{+2} (1 mM)	60
(10 mM)	30
Cu^{+2} (1 mM)	50
(10 mM)	40
Zn^{+2} (1 mM)	39
(10 mM)	65
BSA (20 mg/ml)	250

*Values are expressed relative to control. The specific activity of BP-hydroxylase in the absence of modifier was 0.60 n moles/min/mg protein. Modifiers were pre-incubated with the microsomes for 3 to 5 min at 30°C before the addition of substrate and co-factors.

around 430 nm). But after solubilization this absorption was lost with the concomitant appearance of the absorption peak at 449 and a peak at 420 nm representing denatured form of Cyt P-450 (Fig. 2).

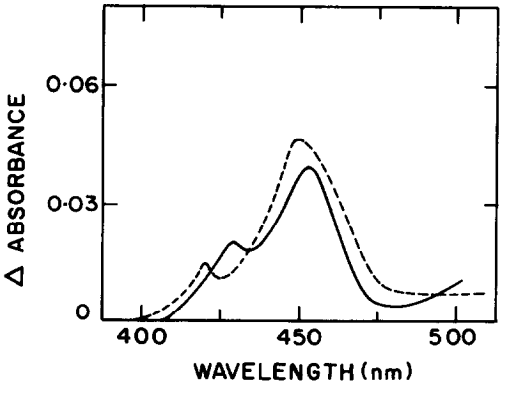


Fig. 2 : Carbon monoxide difference spectrum of microsome (———) and its cholate solubilized preparation (------) from BP treated A.ochraceus TS. The protein concentration was 2 mg/ml and spectrum was recorded in Cary Model 17D spectrophotometer using 3 ml cuvettes.

The response of hydroxylase activity towards metal ions is also consistant with the previous results (6,11). Co^{+2}, Cu^{+2} and Zn^{+2} inhibited the hydroxylation at the concentratiions used (Table II). The only difference being that EDTA (1-5 mM) had no inhibitory effect on this hydroxylation. The stimulatory effect of BSA is possibly due to the increased solubility of BP in aquous reaction mixture. The hydroxylase activity in the microsome decreases by 25-50% after standing at 4° for 24 h while the same stored at -20° retained 60% of the original activity after 3 to 4 weeks. Again, preincubation at 45° for 15 min showed 50-60% less activity than at 30°. Inhibition of NADPH-dependent BP hydroxylase activity by flavone (Fig.1c) showed its similarity with Cyt $P\text{-}450_{LM_6}$ which is produced in rabbit lever after induction with TCDD (13). On the other hand, $NaIO_4$ dependent activity is found to be stimulated at low and inhibited at high concentration respectively. Presumably the flavone may affect the peroxidative function of Cyt P-450 in a dose dependent manner.

The effect of different classical inducer of mammalian Cyt P-450 (Table III) show striking resemblance with those obtained with hepatic microsome and hamster fetus cells (6,11). 3-Methyl cholanthrene, benzo(a)pyrene, β-Napthflavone and other aryl hydrocarbons, used as inducers, enhanced the hydroxylase activity in the range of 80 to 100 μM concentration 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. On the other hand, Phenobarbital, PCB and progesterone did induce the BP hydroxylase to some extent, although a significant induction of NADPH Cyt C reductase activity is observed (3 to 5 fold). It may be mentioned that the inducer added at zero time caused no significant induction and produced a decrease in cell population.

The importance of this reaction in fungi has yet to be determined. However, the results demonstrated that a typical soil fungus has the ability to oxidise BP, an ubiquitous environmental pollutant in the same way as mammalian Cyt P-448 monooxygenase. The metabolic products were separated by the method of Kinoshita et al. (14) and were found to consist of three types of products; BP-diol, quinones and phenols. The determination of relative stereochemistry is in progress. Applications of these enzymes are being thought in carcinogen removal and assay, e.g., removal of BP from some food stuffs or food additives. For this, the use of immobilized

Table III. Effect of inducing agents on different components of BP hydroxylase system in A.ochraceus TS.

Inducer added		BP hydroxylase activity (nmoles/min/mg)	NADPH-Cyt C reductase (nmoles/min/mg)
Benzo(a)pyrene	40 μM	0.37	45.35
	80 μM	0.60	45.75
	100 μM	0.67	46.00
	120 μM	0.64	46.00
	160 μM	0.69	46.00
3-Methyl Cholanthrene	80 μM	0.75	42.00
	100 μM	0.92	45.30
Phenobarbital	100 μM	0.35	150.00
β-Napthaflavone	100 μM	0.62	59.00
Benzanthracene	100 μM	0.67	49.00
Polychlorinated biphenyl (PCB, Arocolor 1254)	100 μM	0.27	115.00
Pyrene	100 μM	0.67	59.00
Naphthalene	100 μM	0.85	49.00
Phenanthrene	100 μM	0.80	48.00
Anthracene	100 μM	0.72	50.00
Progesterone	100 μM	0.37	78.00

microsomal system may prove convenient and appropriate. One problem is the expensive NADPH requirement which has been at present overcome by using $NaIO_4$, where no Cyt P-450 reductase, other enzymes or oxygen are needed. At present methods are also being developed for the large scale rapid isolation of microsomal fraction without recourse to high g centrifuges and some success has been achieved on this problem. Moreover, the results reported here suggest that multiple forms of Cyt P-448/P-450 may exist in A.ochraceus TS and extends the list of properties that the enzymes from this eucaryotic source share with the mammalian system.

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