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The involvement of cytochrome *P*-450 monooxygenase system in aflatoxin biosynthesis by *Aspergillus flavus*

Abidin Bin Hamid and John E. Smith

Applied Microbiology Division, Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow, U.K.

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SUMMARY

Phenobarbital stimulated aflatoxin biosynthesis by *Aspergillus flavus* and this was paralleled by an increase in microsomal NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450 activities. Aflatoxin biosynthesis was inhibited by SKF 525-A, metyrapone and cyanide, inhibitors of the cytochrome *P*-450 monooxygenase system, further suggesting that aflatoxin biosynthesis by *A. flavus* could be mediated by a cytochrome *P*-450 monooxygenase enzyme system.

INTRODUCTION

The involvement of particular enzymes in aflatoxin biosynthesis has been demonstrated by many investigators [22]. Shih and Marth [19] reported that aflatoxin production by *Aspergillus flavus* was increased in the presence of NADPH, while the conversion of sterigmatocystin to aflatoxin by cell-free extracts of *A. parasiticus* was shown to be NADPH-dependent [20] and the enzymes involved were considered to be microsomal [4]. A relatively stable enzyme system that converted versiconal hemiacetal acetate to versicolorin A has been isolated from the homogenized cells of *A. parasiticus* [24].

Monooxygenase enzyme activity involves the integrity of electron flow between co-factor NADPH and cytochrome *P*-450 through NADPH-cytochrome *P*-450 reductase. Fungal polysubstrate monooxygenase enzyme systems have been shown to be involved in the metabolism of a wide range of compounds including steroids, dyes, alkaloids and patulin, and in drug hydroxylation [9,18,21].

Monooxygenases of the cytochrome *P*-450 type are substrate-inducible. Phenobarbital, 3-methylcholanthrene and certain insecticides and polychlorinated biphenyls can stimulate the oxidative activity of mammalian microsomes, and this has been associated with an increase in the microsomal content of monooxygenase enzymes [17].

The presence of polysubstrate monooxygenase activity in the microsomes of *A. parasiticus* has been demonstrated by Bhatnagar et al. [5], together with stimulation of aflatoxin synthesis by phenobarbitone.

Correspondence (present address): A.B. Hamid, Food Technology Division, MARDI, P.O. Box 12301, Kuala Lumpur, 50774, Malaysia.

The present study was conducted to demonstrate the involvement of the cytochrome *P*-450 monooxygenase enzyme system in aflatoxin biosynthesis by *A. flavus* using phenobarbital induction together with the use of selective inhibitors of the monooxygenase enzyme system, viz. 2-diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A) and metyrapone. These latter compounds are believed to inhibit the cytochrome *P*-450 enzyme system by acting as alternative substrates [3].

MATERIALS AND METHODS

Microorganism

Aspergillus flavus 102566 was obtained from the Commonwealth Mycological Institute. Stock cultures were maintained on potato dextrose agar (Oxoid) slants at 4°C.

Inoculum

A. flavus was grown on potato dextrose agar slants at 30°C for 10 days. Spores were harvested with a sterile wetting solution (100 ml distilled water plus three drops of Tween 80), washed three times with sterile distilled water and made up to a final spore concentration of 2×10^6 per ml.

Medium

Sucrose low salts medium was used in these experiments. The medium contained (per litre): 85 g sucrose, 10 g L-asparagine, 3.5 g $(NH_4)_2SO_4$, 10 g KH_2PO_4, 2 g $MgSO_4 \cdot 7H_2O$, 75 mg $CaCl_2 \cdot 2H_2O$, 10 mg $ZnSO_4 \cdot 7H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 5 mg $MnCl_2 \cdot 4H_2O$, 2 mg ammonium molybdate $((NH_4)_6Mo_7O_{24} \cdot 4H_2O)$ and 2 mg disodium tetraborate ($Na_2B_4O_7$).

Culture conditions

100 ml of medium were dispensed into 250-ml wide-mouthed Erlenmeyer flasks supplemented with or without 0.2% phenobarbital (PB), plugged with non-absorbent cotton wool and autoclaved at 110°C (10 p.s.i.) for 20 min. SKF 525-A (2 mM), metyrapone (6 mM) or KCN (10 mM) were added aseptically to flasks after cooling. Flasks were inoculated with 1 ml of inoculum and incubated at 25°C on a Gallenkamp orbital shaker at 200 rpm for 6 days.

Microsomal preparation and enzyme analyses

Cultures of *A. flavus* were chilled in an ice-bath for 20 min and vacuum-filtered. The mycelium was washed three times with cold distilled water and once with cold 0.067 M phosphate buffer, pH 6.9. 50 g mycelial mat were homogenised for 3 min in a chilled high-speed Waring blender (Universal Motor Model 5BA60VL67) with 5 g glass beads (Sigma type V) and chilled 0.067 M phosphate buffer pH 6.9 to give a final concentration of 2 ml buffer/g wet mycelium. The homogenate was centrifuged at 15 000 × *g* to obtain a post-mitochondrial supernatant. This supernatant was recentrifuged at 35 000 × *g* for 3 h to obtain the microsomal pellets. All centrifugations were done at 4°C using an MSE High Speed 18 centrifuge.

Microsomal pellets were solubilised by resuspending at approximately 2 mg/ml in 15 ml of 0.1 M phosphate buffer, pH 7.6, containing 0.01 M EDTA, 0.01 M glutathione, 0.25 M sucrose [11] and 0.5% sodium deoxycholate [7] and stirred in an ice-bath for 30 min. The solution was then centrifuged at 35 000 × *g* at 4°C for 3 h. The supernatant obtained was used for protein analysis [6]. Cytochrome *P*-450 was assayed by the method of Omura and Sato [16] and NADPH-cytochrome *P*-450 reductase was assayed by a modification of the method of Masters et al. [12]. The assay depended upon measurement of the rate of cytochrome *c* reduction at 550 nm. The reaction mixture contained 2.9 ml microsomal protein and 0.2 μmol cytochrome *c* (Sigma, type III) contained in a 1 cm cuvette. The reaction was started by the addition of 0.1 M 0.2% NADPH (Sigma, type I) and the reaction was observed by measuring changes in absorbance at 550 nm against a blank which contained buffer solution minus NADPH.

Analytical determination

For dry weight determinations, the mycelium was harvested by Buchner filtration, washed with distilled water and dried at 70°C for 24 h, cooled in

a desiccator, and weighed.

Aflatoxins from the liquid medium and mycelium were extracted according to the methods of Shih and Marth [19] and Uraih and Chipley [23], respectively. Aflatoxin residues from sample vials were dissolved in 1 ml chloroform, and 10 μl aliquots were spotted on thin-layer chromatography (TLC) plates coated with silica gel G (Merck). Aflatoxin standards of B_1 and G_1 were similarly spotted. The plates were then developed in the solvent system of toluene/ethyl acetate/chloroform/90% formic acid (70:50:50:20). The plates were dried in a fume-cupboard and observed under long wave ultraviolet light (366 nm) in a dark viewing cabinet (Type A, P.W. Allen & Co.). The samples which fluoresced at the same R_f values as the aflatoxin standards were marked and scraped off for quantitative spectrophotometric estimation (Shimadzu UV-120-02) according to the method of Nabney and Nesbitt [15].

Statistical analysis

An analysis of variance [14] was carried out for all results. The differences between the treatment means and the control mean were compared using least-significant analysis at 95% significant level.

RESULTS AND DISCUSSION

The effect of phenobarbital on growth, aflatoxin production and NADPH-cytochrome P-450 reductase activity by Aspergillus flavus

Growth of *A. flavus* was only slightly enhanced in the presence of 0.2% phenobarbital (Table 1). However, there was a marked increase in aflatoxin production with phenobarbital treatment. Aflatoxin B_1 levels increased from the control value of 885.4 μg to 1825.8 μg, while aflatoxin G_1 increased from 782.1 μg to 1630.5 μg (Table 1).

The level of NADPH-cytochrome *P*-450 reductase in the microsomes from the control cultures gradually increased from day 2 to a maximum level of 28.59 μmol cytochrome *c* reduced/min/mg protein at day 6 (Fig. 1). The presence of phenobarbital in the culture medium enhanced the level of NADPH-cytochrome *c* reductase more than 2-fold. A maximum amount of the enzyme of 66.00 μmol cytochrome *c* reduced/min/mg protein was obtained in 6-day-old microsomes.

Fig. 2 shows the carbon monoxide difference spectrum of reduced cytochrome *P*-450 of the solubilised microsomes of *A. flavus*. Two peaks were observed, viz. a small peak at 420 nm and a larger

Table 1

Effect of PB, SKF 525-A, metyrapone and cyanide on growth and aflatoxin production by *A. flavus* incubated at 25°C for 6 days

Each result represents the mean ± S.E.M. of six determinations.

Medium	Mycelial dry weight	Aflatoxin ($\mu g \cdot 100\ ml^{-1}$)		Total $B_1 + G_1$	
	($g \cdot 100\ ml^{-1}$)	B_1	G_1	$\mu g \cdot 100\ ml^{-1}$	$\mu g \cdot g^{-1}$
Control	2.55 ± 0.11	885.4 ± 12.5	782.1 ± 8.5	1667.5 ± 21.1	653.9 ± 17.7
PB (0.2% w/v)	3.10 ± 0.08[b]	1824.8 ± 15.7	1630.8 ± 12.8	3455.6 ± 19.1	1114.7 ± 16.5
SKF 525-A (2 mM)	2.88 ± 0.09[a]	159.5 ± 5.9	128.3 ± 9.8	387.8 ± 15.1	99.9 ± 6.5
Metyrapone (6 mM)	2.04 ± 0.10[b]	591.6 ± 12.2	369.9 ± 8.7	961.5 ± 17.1	471.1 ± 10.8
KCN[c] (10 mM)	2.21 ± 0.09[a]	550.8 ± 8.8	440.7 ± 10.5	991.5 ± 15.7	448.6 ± 11.7

[a] No significant difference ($P > 0.05$) from control.

[b] Significant difference ($P < 0.05$) from control.

[c] Added to 2-day-old cultures to allow spore germination.

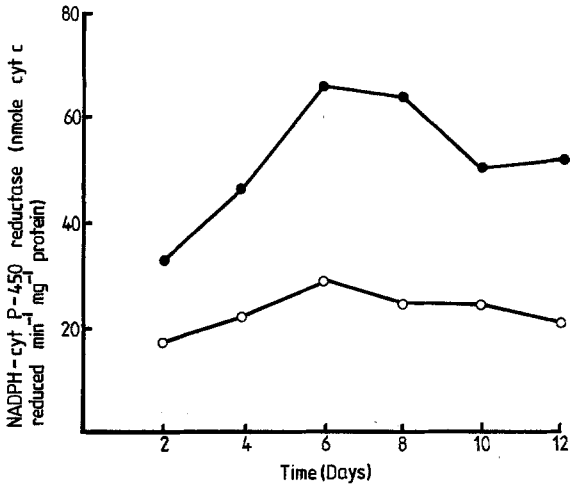

Fig. 1. Effect of phenobarbital on microsomal NADPH-cytochrome *P*-450 reductase from *A. flavus*. ○, control; ●, phenobarbital-treated.

peak at 450 nm. There was an increase in the peak at 450 nm with the PB-treated microsomes, thus substantiating the participation of cytochrome *P*-450 in the biosynthesis of the aflatoxins. The induction mechanism of PB is known to affect several levels of cytochrome *P*-450 monooxygenase [21]. The increase of cytochrome *P*-450 as well as

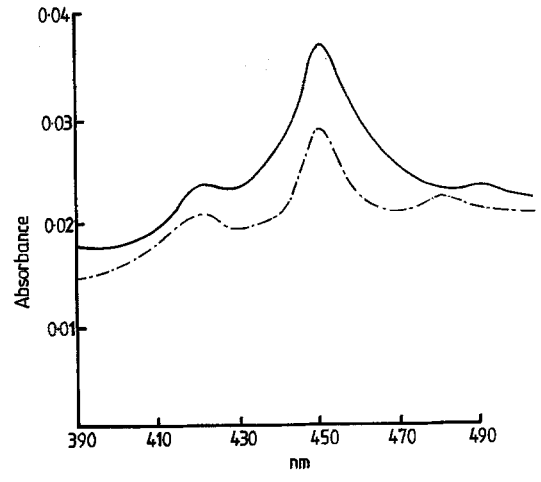

Fig. 2. Carbon monoxide difference spectra of reduced cytochrome *P*-450 of microsomes from *A. flavus*; microsomal protein content 2 mg/ml. ———, control; ----, phenobarbital-treated.

NADPH-cytochrome *P*-450 reductase was paralleled by an increase in aflatoxin biosynthesis of the fungus, this being in substantial agreement with the previous observations for *A. parasiticus* [5].

The increase in aflatoxin levels with phenobarbital treatment in the present results and with *A. parasiticus* [5] could be due to the increase in the activity of the monooxygenase enzyme system, since the hydroxylation of the pathway intermediates is a crucial step in aflatoxin biosynthesis. Conversion of versiconal hemiacetal acetate to versicolorin A [24] and sterigmatocystin to aflatoxin [20] could involve monooxygenase activities and, thus, could be stimulated by phenobarbital. Phenobarbital has been shown to stimulate trisporic acid production of *Blakeslea trispora* [8] and alkaloid production by *Claviceps purpurea* [1,2], and increased polysubstrate monooxygenase activity was demonstrated in each case.

The effect of SKF 525-A, metyrapone and cyanide on growth and aflatoxin production by Aspergillus flavus

SKF 525-A (2 mM) and KCN (10 mM) did not significantly affect growth of *A. flavus* over the experimental period. In contrast, metyrapone (6 mM) did result in a small but significant decrease in growth (Table 1).

All compounds significantly reduced aflatoxin production. The effect was most marked in the presence of SKF 525-A, with approximately 83% inhibition of total aflatoxin synthesis. Metyrapone and KCN caused 42% and 40% inhibition, respectively (Table 1). Similar inhibitory patterns were observed in the hydroxylation of benzo(a)pyrene by a microsomal preparation of *Aspergillus ochraceus* [10,11] and in the demethylation of pisatin (an antimicrobial compound produced by infected pea tissue) by *Nectaria haematococca* [13]. Cyanide also strongly inhibited the demethylation reaction in intact cells of the fungus [13].

The present results strongly implicate the involvement of the cytochrome *P*-450 monooxygenase enzyme system in aflatoxin biosynthesis by *A. flavus*.

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