APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1987, p. 1711–1713
0099-2240/87/071711-03$02.00/0

Vol. 53, No. 7

Appearance of Enzyme Activities Catalyzing Conversion of Sterigmatocystin to Aflatoxin B_1 in Late-Growth-Phase *Aspergillus parasiticus* Cultures

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Two activities involved in terminal pathway conversion of sterigmatocystin to aflatoxin B_1 were isolated from an aflatoxin-nonproducing mutant of *Aspergillus parasiticus* (avn-1), and the time course of appearance of the activities in culture was determined. Subcellular fractionation of fungal mycelia resolved the two activities into a postmicrosomal activity which catalyzed conversion of sterigmatocystin to *O*-methylsterigmatocystin and a microsomal activity which converted *O*-methylsterigmatocystin to aflatoxin B_1. The two activities were absent in 24-h-old cells, increased to optimum levels during the stationary phase, and then declined.

Early elucidation of the mechanisms of alfatoxin biosynthesis centered on establishment of intermediates in the pathway (23). The identities and sequence of production of several intermediates were determined by feeding radiolabeled precursors to resting mycelium of wild-type *Aspergillus parasiticus* isolates or pathway-blocked mutants (21). Regulation of the pathway involves complex controls which are strongly influenced by specific cellular events in the growth cycle (6–10, 15–17, 19).

Preliminary studies demonstrated that cell-free preparations of *A. parasiticus* or *Aspergillus flavus* could catalyze conversion of certain pathway intermediates to aflatoxin B_1 (AFB_1) (2, 3, 11, 14, 18, 20, 24, 25). Both intact cells (13) and cell extracts (14, 20) of *A. parasiticus* catalyzed conversion of sterigmatocystin (ST) to AFB_1. A recent study using intact cells placed *O*-methylsterigmatocystin (OMST) between ST and AFB_1 (5), as shown in Fig. 1. A preliminary report from our laboratory indicated that ST-to-AFB_1 conversion requires at least two enzymatic activities present in mycelial extracts (A. R. Lax, T. E. Cleveland, D. Bhatnagar, and L. S. Lee, Plant Physiol. **80**[Suppl.]:19, 1986).

The present investigation established the time course sequence of the appearance and disappearance of enzymes involved in the conversion of ST to AFB_1 by using a mutant of *A. parasiticus* blocked at an earlier step in the biosynthetic pathway.

The *A. parasiticus* mutant strain (avn-1) (SRRC 163; ATCC 56774) used in this study does not produce aflatoxin but accumulates averantin (4). avn-1 was maintained on potato glucose agar slants, and spores were produced on potato glucose agar petri plates. Spores were transferred from plates into sterile, deionized water containing 0.04% Triton X-100 and diluted to obtain about 2×10^5 viable spores ml^{-1}. Liquid growth media (1), 100 ml in 250-ml Erlenmeyer flasks, were inoculated with 1 ml of spore suspension. Cultures were shaken (100 rpm) at 28°C, and fungal mycelia were harvested at various incubation times by vacuum filtration on Miracloth (Calbiochem-Behring, La Jolla, Calif.). Mycelia were washed with extraction buffer (0.2 M sodium phosphate, pH 7.5) for cell-free enzyme assays. Excess wash solution was removed by vacuum filtration, leaving a damp cake of mycelia for fresh- and dry-weight determinations.

Cell extracts were prepared by grinding chilled mycelia (1.5 g), from the same cultures used for intact-cell assays, with sea sand in a mortar and pestle in 2.5 ml of extraction buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.). The macerate was filtered through Miracloth and centrifuged (at 4°C) in a J2-21 centrifuge with a JA 20 rotor (Beckman Instruments, Inc., Fullerton, Calif.), first at $5,000 \times g$ (pellet discarded) for 10 min and then at $20,000 \times g$ for 15 min. The $20,000 \times g$ supernatant was further fractionated by centrifugation at $105,000 \times g$ for 90 min with an RC70 ultracentrifuge with a T875 rotor (Du Pont Co., Wilmington, Del.). The $105,000 \times g$ supernatant was carefully removed, and the pellets were suspended in an equal volume (equal to that of the supernatant) of 0.1 M sodium phosphate, pH 7.5, containing 10% (vol/vol) glycerol. For enzyme assays, 1 ml of the $20,000 \times g$ supernatant (cell-free fraction), the $105,000 \times g$ supernatants (postmicrosomal fraction), or the $105,000 \times g$ pellet (microsomal fraction) was added to 2.5 ml of assay buffer (2.15 ml of 0.1 M sodium phosphate, pH 7.5, and 0.35 ml of glycerol) containing 0.5 mM NADPH (Sigma) in 50-ml Erlenmeyer flasks. Enzyme reactions were initiated by addition of substrate (35 μg of ST or OMST; Sigma) in 35 μl of acetone, followed by 2 h of incubation at 28°C with gentle shaking (60 cycles min^{-1}). Reactions were terminated by shaking with 20 ml of $CHCl_3$ for 15 min. The $CHCl_3$ phase was removed, and the remaining aqueous phase was washed with an additional 10 ml of $CHCl_3$; organic phases were combined and evaporated to dryness on a steam plate. Dried residue was solubilized in 2 ml of acetone, transferred to 2-ml vials, dried under N_2, and dissolved in 50 μl of acetone. A 25-μl portion of the acetone solution was applied as a spot on a thin-layer chromatography (TLC) plate (20 by 20 cm by 0.25 mm, Silica Gel G; Merck & Co., Inc., Rahway, N.J.). TLC plates were developed in a diethyl ether-methanol-

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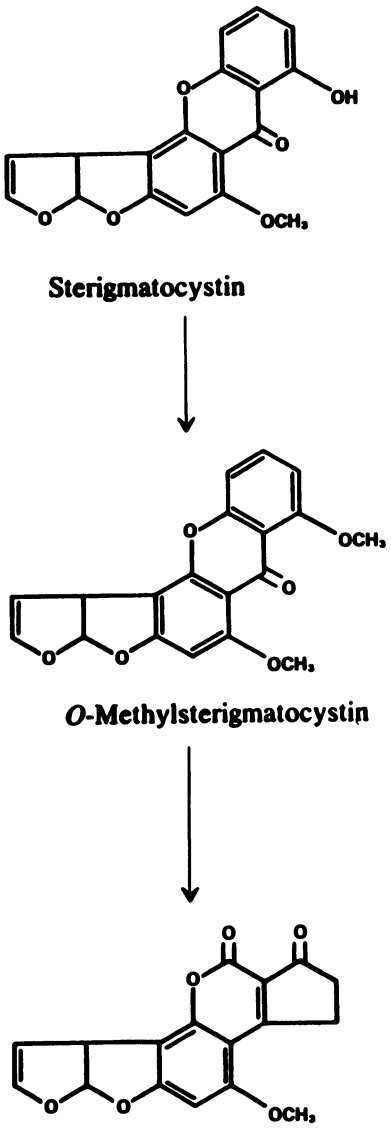

FIG. 1. Reactions occurring during ST-to-AFB_1 conversion.

water (96:3:1 [vol/vol]) solvent system that effectively separated AFB_1 (R_f, 0.37), OMST (R_f, 0.44), and ST (R_f, 0.97). Lanes on TLC plates were scanned for fluorescence with a TLC plate scanner (CS-930; Shimadzu, Kyoto, Japan) at 365 nm for AFB_1 or at 310 nm for OMST (ST was not scanned). Quantitation was accomplished by comparing peak heights of samples and known quantities of authentic standards (Sigma). An alternative TLC solvent system (toluene-ethyl acetate-acetic acid, 50:30:4 [vol/vol]; S. P. McCormick, personal communication) was used for verification of AFB_1 and OMST (R_fs, 0.17 and 0.27, respectively). In some tests, TLC plates were developed and sprayed with 20% (wt/vol) aluminum chloride in ethanol to test for the characteristic changes in intensity and color of fluorescence of ST and OMST (14, 22). OMST demonstrated a characteristic pale blue fluorescence under UV illumination. Fluorescence of this compound intensified and turned yellow-green when sprayed with aluminum chloride. ST exhibited brick-red fluorescence under UV light; this changed to yellow when the plate was sprayed with aluminum chloride. No reaction occurred upon treatment of AFB_1 with aluminum chloride. Soluble protein was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.).

Cell extracts (supernatants after centrifugation at 20,000 × *g*) of 72-h-old mycelia converted ST to two major compounds that were identified and verified by TLC as OMST and AFB_1. Relatively large amounts of residual substrate (ST) from both intact-cell and cell-free assays were visually apparent as yellow fluorescent bands after spraying of TLC plates with aluminum chloride.

Enzymatic activities producing OMST and AFB_1 from ST were completely resolved by centrifugation into microsomal and postmicrosomal fractions. Postmicrosomal fractions catalyzed ST-to-OMST conversion, and microsomal fractions contained an activity converting OMST to AFB_1 (Fig. 2). Both activities were absent in 24-h-old cells, reached optimum levels during the early stationary growth phase of the cultures, and then declined. The microsomal fraction-mediated OMST-to-AFB_1 conversion demonstrated an absolute requirement for NADPH at all time periods tested.

The enzyme activities described in the present investigation contain both methyltransferase (catalyzing ST-to-OMST conversion) and oxidoreductase (catalyzing OMST-to-AFB_1 conversion) components, which are involved in terminal pathway conversion of ST to AFB_1 (Fig. 1). A previous investigation (14) demonstrated that the ST-to-OMST enzymatic reaction is stimulated in the presence of *S*-adenosylmethionine, indicating methyltransferase activity. The absolute requirement of NADPH for OMST-to-AFB_1 conversion observed in the present study strongly indicates oxidoreductase activity. Both activities are regulated by events occurring during late-stage mycelial growth in *A. parasiticus* cultures (Fig. 2), as are enzymes catalyzing production of other secondary metabolites (17). The regulatory mechanism is not eliminated by a genetic block early in the aflatoxin biosynthetic pathway. This is the first report on the time sequence of the appearance and disappearance of specific enzyme activities involved in aflatoxin synthesis by developing *A. parasiticus* cells.

The *A. parasiticus* mutant used in this study accumulates averantin, an early intermediate in the pathway, but essentially none of the subsequent pathway intermediates. This suggests that intermediates after averantin do not have a major role in the induction of activities catalyzing ST-to-AFB_1 conversion in the final stages of the pathway. The decrease in activities observed in older cultures may reflect cessation of synthesis and subsequent decay of enzyme proteins, possibly by proteases (17). The possibility that feedback inhibition by enzyme products (17) past averantin in the pathway contributes to the observed decline in activities probably can be excluded since the mutant used in this investigation is blocked at two steps (averantin to averufin and versicolorin A to ST) (4).

The period between 48 and 72 h of fungal growth in this study included the transitional phase leading into the stationary growth phase (Fig. 2), during which AFB_1 begins to accumulate in a native *A. parasiticus* isolate (8). In a previous investigation, addition of cycloheximide before this transition period stopped AFB_1 synthesis; addition after this period resulted in continued AFB_1 synthesis (8). The results suggested that proteins catalyzing AFB_1 synthesis were produced de novo during the transitional period. In another

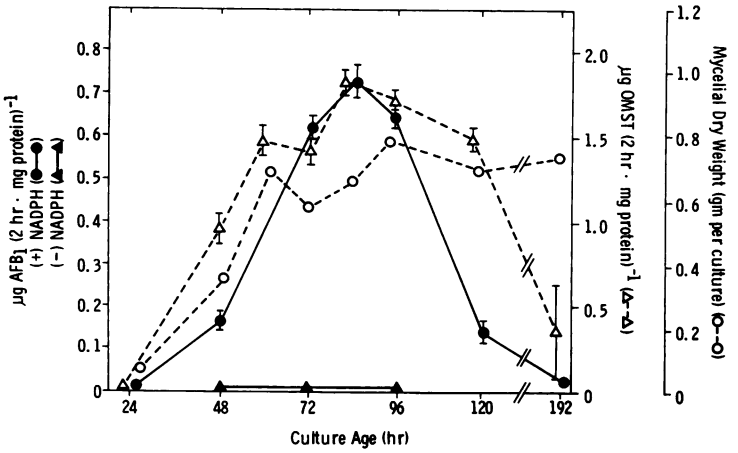

FIG. 2. Production of AFB_1 and OMST (2 h · mg of protein)$^{-1}$ in the presence of microsomal and postmicrosomal fractions, respectively. Vertical bars show the range about the means of duplicate assays.

investigation (12), with a wild-type fungal isolate and culture conditions very closely approximating those of this study, AFB_1 accumulation at high levels began after 48 h. These results correlate well with the times of maximal increases in ST-to-OMST and OMST-to-AFB_1 conversion activities in this study.

We acknowledge S. P. McCormick for valuable advice and A. G. Wilfred for technical assistance.

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