Secondary biosynthesis of aflatoxin B₁ in *Aspergillus parasiticus*

R. W. DETROY and C. W. HESSELTINE

*Northern Regional Research Laboratory, Peoria, Illinois*

Received October 22, 1969


The effect of two inhibitors on the formation of aflatoxin B₁ synthetase activity in strain NRRL 2999 *Aspergillus parasiticus* has been studied. Aflatoxin B₁ synthesizing activity was measured in vivo by incorporation of the ¹⁴C-methionine methyl group into aflatoxin B₁. Cycloheximide at a concentration of 150 µg/ml blocks protein synthesis completely. If addition of cycloheximide is made before B₁ synthetase appears, no activity accumulates; if added during accumulation, activity is frozen at the level reached at the time of addition. The cycloheximide effect is reversible since morphogenesis, total protein synthesis, and aflatoxin B₁ synthetase activity all resume after removal of the inhibitor.

DL-p-Fluorophenylalanine partially inhibits aflatoxin B₁ synthesis in vivo; however, its effect upon macromolecular synthesis is incomplete even at high concentration levels. Once formed, the aflatoxin synthetase appears to maintain B₁ synthesis when further protein synthesis is blocked; i.e., it is not rapidly degraded.

Introduction

A large body of information, summarized by Richards and Hendrickson (11), supports the suggestion by Lynen and Tada (9) that many secondary metabolic substances are derived by the condensation of acetate and malonate units. A plausible mechanism would be similar to that of fatty acid biosynthesis (13) except that the intermediate polyketo thioesters would remain either completely or partially unreduced during the condensation. In support of this mechanism, cell-free extracts have been reported which incorporate acetyl or malonyl thioesters into 6-methylsalicylic acid (9), patulin (1), stipitatic acid (12), and alternariol (7). Others (2) have also shown that 13 of 17 carbon atoms of aflatoxin B₁, a mold metabolite, are ¹⁴C-acetate derived.

Many microorganisms, particularly fungi, elaborate large quantities of compounds that have no apparent function in the general metabolism of the cell. These compounds have been termed secondary metabolites (3), such as those produced by *Penicillium* sp.; namely, 6-MS, patulin, and numerous other antibiotics. Bu'Lock et al. (6), studying 6-methylsalicylic acid synthesis, demonstrated a shift in the metabolism from a balanced phase of growth (tropophase) to a stationary or unbalanced phase (idiophase) associated with 6-methylsalicylic acid synthesis.

This metabolic phasing is characteristic of many fungal systems.

Because of the carcinogenicity of aflatoxin B₁, we believed it would be appropriate to study the macromolecular events preceding the secondary biosynthesis of aflatoxin B₁ in *Aspergillus parasiticus*. This report describes the effect of protein synthesis inhibitors (cycloheximide and fluorophenylalanine) on the appearance of aflatoxin B₁ synthetase activity during the transitional metabolic phase of growth, a method for measuring in vivo protein synthesis, and the timing of aflatoxin B₁ biosynthesis in *A. parasiticus* NRRL 2999.

Materials and Methods

Culturing Methods

*A. parasiticus* NRRL 2999 was maintained at room temperature on potato dextrose agar slants (20 g dextrose, 0.2 g CaCO₃, 0.2 g MgSO₄, 200 g potatoes, and 1 liter of distilled water) with transfers every 10 days. Spores from individual slants were used to inoculate 100 ml of germinating medium 2% YES (200 g sucrose, 20 g yeast extract, 0.5 g MgSO₄·7H₂O, 0.005 g ZnSO₄, 0.1 g FeSO₄, and 1 liter of distilled H₂O) contained in a 500-ml Erlenmeyer flask. Growth at 30°C on a rotary shaker generally produced about 1–3 g (wet weight) of mycelial pellets harvested by filtration.

The filtered mycelia were washed with YES medium, and weighed samples (wet weight) were suspended in 5 ml of 1% YES medium (200 g sucrose, 10 g yeast extract, 0.5 g MgSO₄·7H₂O, 0.005 g ZnSO₄, 0.1 g FeSO₄, and 1 liter of distilled H₂O) contained in 50-ml test tubes. Various ¹⁴C-biochemicals and inhibitors were included.

1 A laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

2 Abbreviations used: YES, yeast extract–sucrose; FPA, fluorophenylalanine; t.l.c., thin-layer chromatography.
Table I shows the effect of FPA on mycelial growth and aflatoxin B₁ synthesis. Aflatoxin B₁ was not detectable before 48 h growth; however, aflatoxin synthesis was observed at 68 h. In the presence of 200 µg/ml FPA, added at 24 h, mycelium incubated for 90 h produced only 32 µg of B₁ compared to 130 µg for a 90-h control incubation. However, cell mass increase was not completely blocked by addition of inhibitor at 24 h. The addition of FPA (200 µg/ml) at 48 or 68 h growth had no effect upon total aflatoxin synthesis after 90 h incubation, although cell mass increase was interrupted. These preliminary data with FPA indicated a possible requirement for new protein synthesis during the transition period for B₁ synthesis; however, FPA does not completely block cell growth, which is required to measure in vivo protein synthesis and synthetic capacity.

Effect of Cycloheximide on Protein Synthesis and B₁ Synthetase Activity
In the preliminary evaluation of protein-synthesizing inhibitors, cycloheximide proved to be most effective. The amino acid analogue of phenylalanine, FPA (200 µg/ml), inhibited B₁ synthesis by 89–90% under growing cell conditions. Initial studies were carried out using FPA to determine if new protein synthesis is required during the transitional phase for aflatoxin B₁ synthesis.

Table I: Effect of FPA on mycelial growth of *Aspergillus parasiticus* and aflatoxin B₁ synthesis

<table>
<thead>
<tr>
<th>Addition of FPA, µg/ml</th>
<th>Mycelial harvest time, h</th>
<th>Mass dry weight, g</th>
<th>Aflatoxin B₁, µg/growth flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.31</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.35</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>0.60</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.80</td>
<td>139</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.42</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.60</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>0.70</td>
<td>130</td>
<td></td>
</tr>
</tbody>
</table>
effective (Fig. 2). After 12 h of incubation, addition of 150 μg/ml cycloheximide completely blocked cell growth and protein synthesis. As a routine assay, we have looked at the appearance of in vivo aflatoxin synthetase activity in 24- to 30-h mycelial pellets transferred to a fresh 1% YES medium. Figure 3 shows that the addition of 150 μg/ml cycloheximide to 40-h mycelial pellets (zero time) inhibits the incorporation of 14C-(CH₃)-methionine into trichloroacetic acid insoluble material and the 14C-CH₃O-group into aflatoxin B₁. With no inhibitor present, aflatoxin B₁ synthesis was detectable at 67 h, a time which corresponded to the transitional phase of growth. This experiment also demonstrated that 14C-methionine incorporation into protein paralleled increase in dry weight and that the method was satisfactory.

When we used cycloheximide selectively through the developmental phase of the organism, Table II illustrates the point in the transitional growth phase where de novo protein synthesis was required for aflatoxin B₁ synthesis using cycloheximide.

**TABLE II**

<table>
<thead>
<tr>
<th>Cycloheximide addition</th>
<th>14C protein synthesis, c.p.m.</th>
<th>Total incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, h</td>
<td></td>
<td>14C Protein synthesis, c.p.m.</td>
</tr>
<tr>
<td>40</td>
<td>10⁻³</td>
<td>75</td>
</tr>
<tr>
<td>47</td>
<td>19⁻³</td>
<td>75</td>
</tr>
<tr>
<td>60</td>
<td>20⁻³</td>
<td>75</td>
</tr>
<tr>
<td>65</td>
<td>21⁻³</td>
<td>75</td>
</tr>
<tr>
<td>78</td>
<td>23⁻³</td>
<td>87</td>
</tr>
</tbody>
</table>

*The amount of 14C-methionine incorporated at the indicated time of cycloheximide addition.†Total 14C-methionine incorporated at time of harvest. The total incubation time refers to the activities recorded at the respective harvest times.‡Incorporation of 14C-(CH₃) of methionine into aflatoxin B₁.

---

**Fig. 2.** Effect of cycloheximide on protein synthesis and cell growth.

**Fig. 3.** The effect of cycloheximide on incorporation of 14C-methionine into hot trichloroacetic acid insolubles in vivo and the appearance of B₁ synthetase activity by Aspergillus parasiticus. Mycelial pellets (1.0 to 1.5-mg aliquots, dry weight) from a 24- to 40-h germinating medium culture of strain NRRL 2999 were transferred to test tubes containing 1.25 μcurie 14C-(CH₃)-methionine in a 5-ml volume of 1% YES medium. After this addition, 150 μg of a cycloheximide solution was added while an appropriate volume of H₂O was added to the control tubes. Incubations were carried out at 30°C for the indicated times and terminated. The mycelial pellets were washed by filtration, oven-dried for 30 min, and weighed. The dry mycelium samples were ground with 60-mesh glass beads to a fine powder. The material was diluted with 30 ml of H₂O, plus 5 ml of 5% trichloroacetic acid. Tubes were heated at 85°C for 10 min and allowed to cool. Insoluble material from each tube was centrifuged out at 9000 X g for 10 min and washed twice with 10 ml of 95% ETOH. The pellet material was transferred to scintillation vials and assayed for radioactivity in a scintillation counter after the addition of 15 ml of Bray's scintillator solution. Aflatoxin B₁ synthesis was measured according to methods previously described. ○ D.W. mass; ■ B₁ synthesis; Δ 14C-protein.

**Fig. 4.** Reversible inhibition of protein synthesis by cycloheximide. I, control; II, cycloheximide added at 50 h; III, cycloheximide removed at 70 h.
synthesis is required to make the aflatoxin synthetase enzymes. Cycloheximide addition at 60 h, or even before, blocks protein synthesis immediately as measured by $^{14}$C-methionine incorporation and any subsequent aflatoxin synthesis. This indicated a requirement for new enzyme synthesis at the transitional phase for aflatoxin appearance. Although the addition of cycloheximide at 65 h or later blocks protein synthesis completely, some synthesis of $B_1$ synthetase activity occurs between 60 and 65 h.

Reversible Inhibition of Protein Synthesis by Cycloheximide

Mycelial pellets were sampled at various times after cycloheximide addition and washed with 100–150 ml of distilled H$_2$O by filtration. The pellets were then resuspended in fresh 1% YES medium, plus $^{14}$C-methionine, and assayed for protein synthesis. Figure 4 shows that complete inhibition of amino acid incorporation was virtually immediate upon cycloheximide addition. Furthermore, the resumption of incorporation after removal of cycloheximide (20 h later) was also achieved immediately and rose to the same level as the control within 22 h. Aflatoxin $B_1$ synthesis also resumed after removal of cycloheximide.

Discussion

Studies of culture conditions that lead to high levels of production have been extensive, and many times these have shown that the synthesis of secondary metabolites is associated with a stationary or resting phase of the culture (3). This phase can sometimes be approximated by transfer of cultures to resting-cell media which can support no further growth.

For aflatoxin $B_1$, we have demonstrated that a shift in the metabolic capacity of A. parasiticus from a logarithmic phase of growth (tropophase) to an unbalanced phase (idiophase) is associated with aflatoxin biosynthesis. The effect of cycloheximide on protein synthesis and the production of aflatoxin $B_1$ indicates that the $B_1$ synthetase is produced late in the fermentation; i.e., during growth in the idiophase. Once formed, the enzymes maintain aflatoxin $B_1$ synthesis when further protein synthesis is blocked; i.e., the system is not rapidly degraded.

On removal of cycloheximide after preincubation of the fungal pellets with the antibiotic, the organism still has the ability to carry out protein biosynthesis and eventually aflatoxin $B_1$ synthesis. Thus the exposure of the organism to cycloheximide blocks macromolecular synthesis; however, the antibiotic is not lethal to the organism. The reversibility effect by cycloheximide removal would indicate the drug is not binding tightly to any tRNA moiety or mRNA-ribosome site. The antibiotic might possibly be inhibiting protein synthesis by competing for or saturating available tRNA sites on the mRNA-ribosome complex. Since the aflatoxin $B_1$ synthetase is not formed constitutively as demonstrated, we must ask what controls its formation until the idiophase begins. A number of investigators have shown that the end of tropophase is marked by sharp changes in the availability of many metabolic intermediates at the end of tropophase (4, 5), including the pools of acetyl- and malonyl-CoA moieties that are incorporated into aflatoxin $B_1$, which may possibly influence secondary enzyme formation at the transitional period.

For secondary metabolites particularly, such as aflatoxin $B_1$, formed from acetyl- and malonyl-CoA moieties, the new enzyme system is the one that assembles acetyl-CoA and malonyl-CoA units into a polyketide chain, stabilized by cyclization. The early enzyme activities are those of fatty acid synthesis and are already present. Malonyl-CoA thus would become the substrate most likely to elicit enzyme induction. Since the formation of malonyl-CoA from acetyl-CoA is the limiting reaction in fatty acid synthesis, then malonyl-CoA would be limited; however, citrate has been shown to stimulate this reaction. Therefore, a sudden buildup of tricarboxylic acid intermediates, which occurs at tropophase termination (4, 5), could lead to a novel buildup of malonyl-CoA to trigger secondary enzyme induction. This type of mechanism, however, would also demand some factor for limitation of fatty acid synthesis.

Although the inhibitor effects upon the aflatoxin $B_1$ synthesizing enzymes gives one useful insight on possible biosynthetic control and regulation, available data are still insufficient to explain a mechanism for possible substrate induction or derepression of secondary biosynthetic enzyme synthesis. It is hoped that a cell-free system can now be obtained to test the various metabolic control parameters needing investigation.
Acknowledgment

The technical assistance of Mrs. Melba Milburn is gratefully acknowledged.