Mevinolinic Acid Biosynthesis by *Aspergillus terreus* and Its Relationship to Fatty Acid Biosynthesis

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Mevinolinic acid, the open acid form of mevinolin, which is a metabolite of *Aspergillus terreus*, has been shown to be a potent competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (Alberts et al., Proc. Natl. Acad. Sci. U.S.A. 77:3957-3961, 1980). The biosynthesis of mevinolinic acid was studied by examining the incorporation of [1-14C]acetate and [methyl-14C]methionine into the molecule. These isotopes were rapidly incorporated into mevinolinic acid, with [1-14C]acetate and [methyl-14C]methionine incorporation being linear for at least 10 and 30 min, respectively. A comparison of acetate incorporation into mevinolinic acid and fatty acids indicated that mevinolinic acid biosynthesis increased with a maximum between days 3 and 5 of growth; at this time cell growth had ceased and fatty acid biosynthesis was negligible. Hydrolysis of the mevinolinic acid and isolation of the products showed that [1-14C]acetate and [methyl-14C]methionine were incorporated into the 2-methylbutyric acid side chain as well as into the main (alcohol) portion of the molecule.

Mevinolinic acid, a fungal metabolite isolated from cultures of *Aspergillus terreus*, has been found to be a potent competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase.

**MATERIALS AND METHODS**

*A. terreus* ATCC 20542 (Merck strain MF4845) was obtained from the Merck cell culture collection. Standard analytical-grade reagents were used in the preparation of media, and high-pressure liquid chromatography (HPLC)-grade solvents were used for the HPLC analyses. Sodium [1-14C]acetate (2.8 mCi/mmole) and L-[methyl-14C]methionine (2.8 mCi/mmole) were obtained from New England Nuclear Corp., Boston, Mass.

**Instrumentation.** Chromatography was performed with a Beckman model 332 liquid chromatograph system coupled to a Hitachi model 100-40 spectrophotometer, a Spectrophysics model 4100 computing integrator, and a Radiomatic Flo-One model HP radioactive flow detector. An Ultrasil ODS column (4.6 by 250 mm, 10-μm bead) was used for all of the HPLC analyses.

**Culture condition.** Primary seed cultures of *A. terreus* were grown in stationary cultures at 25°C for 3 weeks in 250-ml Erlenmeyer flasks containing 40 ml of KF medium (10 g of glucose, 5 g of corn steep liquor, 40 g of tomato paste, 10 g of oat flour, 10 mg of FeSO4 · 7H2O, 10 mg of MnSO4 · H2O, 0.25 mg of CuCl2 · 2H2O, 1 mg of CaCl2 · 2H2O, 0.56 mg of H3BO4, 0.19 mg of (NH4)2MoO4 · 2H2O, 4H2O, 2 mg of ZnSO4 · 7H2O per liter of medium [pH 7.0]). At the end of this period an additional 40 ml of KF medium was added to the flask, and the cells were stored at 4°C until used. Secondary cultures were prepared by adding 1 ml of primary seed culture (which had been shaken to disperse the spores and mycelia) to 40 ml of KF medium and were incubated at 28°C for 24 h in a model G-24 incubating shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 365 cycles/min. All of the studies on the biosynthesis of mevinolinic acid were performed by adding 1 ml of the secondary seed culture to a 250-ml Erlenmeyer flask containing 40 ml of production medium (45 g of glucose, 24 g of peptonized milk, 2.5 g of yeast extract per liter of medium [pH 7.2]). The suspension was incubated in the New Brunswick shaker as described above. The radioisotope was added to the production cultures, the incubation was continued for an additional 10 min with the sodium [1-14C]acetate or 30 min with the L-[methyl-14C]methionine to allow incorporation of the isotope, and the reaction was stopped by homogenizing the cells at high speed with 40 ml of ethyl acetate for 30 s in a polytron homogenizer. Isotope (10 μCi) was added per 40 ml of incubation culture.

**Identification of mevinolinic acid.** Mevinolinic acid was...
assayed by HPLC of the ethyl acetate extract from the cells. The sample in ethyl acetate was dried down and redissolved in acetonitrile to give a 10-fold concentrate. A 10- to 200-μl sample was injected onto the HPLC column equilibrated with 35% acetonitrile–10 mM phosphoric acid. The flow rate was 2 ml/min. After 3 min at the above solvent composition, a linear gradient to 80% acetonitrile was run over a 12-min period. Mevinolinic acid was detected by its absorbance at 210 nm, and its mass was determined by comparison with a standard calibration curve; 1 μg can be accurately detected. The free-acid form of mevinolin eluted at 11.15 min, with the lactone following at 12.5 min.

Lipid analysis. After addition of [1-14C]acetate to the cells for 10 min, lipids were extracted by the method of Bligh and Dyer (3). The chloroform fraction was concentrated, and a sample was taken to dryness and treated with 0.625 N ethanolic KOH for 30 min at 85°C. Water was added, and the basic solution was extracted with petroleum ether. The aqueous solution was acidified with HCl and extracted again with petroleum ether.

Both the nonsaponifiable and saponifiable fractions were washed with dilute HCl, concentrated, and chromatographed on silica gel G thin-layer plates. The developing solvent was petroleum ether-diethyl ether-acetic acid (75:25:1). The lipids were visualized with iodine vapor; areas corresponding to standard oleic acid and ergosterol were scraped into scintillation vials, scintillation fluid was added, and the contents of the vials were counted in a Packard scintillation counter. Ergosterol was identified as the major sterol component by gas chromatography on a 3% OV-17 column at 280°C.

Assay of fatty acid synthetase and citrate cleavage enzyme. A. terreus cells were collected by filtration at the indicated times. The cells were suspended in phosphate buffer (100 mM potassium phosphate [pH 6.5], 5 mM EDTA, 1 mM β-mercaptoethanol) and broken in a Braun homogenizer with glass beads. The mixture was centrifuged at 15,000 × g for 20 min, and the supernatant was decanted and centrifuged again at 100,000 × g for 60 min. The resulting supernatant was assayed for fatty acid synthetase and citrate cleavage enzyme by the methods of Lyen (6) and Spencer and Lowenstein (8), respectively.

Hydrolysis of mevinolinic acid. The 2-methylbutyrate side chain was prepared by refluxing the radioactive mevinolinic acid (usually about 10 mg) with 10 ml of 0.826 M LiOH for 18 h under nitrogen. The products of the hydrolysis, 2-methylbutyric acid, des-(2)-S-methylbutyryl mevinolinic acid, and unhydrolyzed mevinolinic acid, were isolated by cooling the mixture to 0°C, acidifying it carefully with HCl, adding saturating amounts of NaCl, and extracting with diethyl ether. The potassium salt of 2-methylbutyric acid was formed by the addition of 0.5 ml of 0.625 N KOH in ethanol. After the ether extract was dried, the residue was dissolved in 0.4 ml of acetonitrile and the solution was carefully acidified with HCl. The KCl precipitate was removed by centrifugation, and this acetonitrile solution was used for thin-layer chromatography and for preparation of the bromphenacyl ester of 2-methylbutyric acid.

Thin-layer chromatography was carried out by drying a sample of the acetonitrile solution on a silica gel GF thin-layer plate. The developing solvent was methylene chloride-acetic acid (85:15), and visualization was by UV light.

Bromphenacyl esterification of 2-methylbutyric acid. (i) Preparation. Bromphenacyl esters of the hydrolysis reaction products were made by the method of Pei et al. (7). This solution was used for subsequent analysis by HPLC and gas chromatography. Bromphenacylation was also carried out with standard 2-methylbutyric acid.

(ii) HPLC of bromphenacyl esters. HPLC of the bromphenacyl esters of the hydrolysis reaction products was performed on the Ultrasil ODS column with a flow rate of 2 ml/min. The solvent was 65% aqueous methanol, and detection was by UV absorbance at 254 nm.

RESULTS AND DISCUSSION

The incorporation of [1-14C]acetate into mevinolinic acid by 3-day-old cultures of A. terreus was linear for 10 min. The amount of acetate incorporated into mevinolinic acid varied from culture to culture from as low as 1 to almost 8% of the total added acetate. Unlabeled acetate (or methionine) was not present in the media of 3-day-old cultures, and so there was no dilution of the isotopes when added to the cultures.

The synthesis of mevinolinic acid as well as sterols and fatty acids as a function of the age of the culture is shown in Fig. 2. Incorporation of [1-14C]acetate into mevinolinic acid increased for the first few days, reached a plateau between days 3 and 5, and then declined. The amount of mevinolinic acid present in the cultures increased as the rate of synthesis increased, reached a plateau at day 5, when incorporation of acetate decreased, and was stable for at least 8 days, suggesting that once it was produced, it was not further metabolized. The incorporation of radioactive acetate into cellular lipids is also shown in Fig. 2. In contrast to the

FIG. 1. Structure of mevinolin and mevinolinic acid.
TABLE 1. Incorporation of [1-14C]acetate and [methyl-14C]methionine into the hydrolysis products of mevinolinic acid<sup>a</sup> 

<table>
<thead>
<tr>
<th>Product</th>
<th>Incorporation (nmol) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[methyl-14C]methionine</td>
</tr>
<tr>
<td>Des MB-mevinolinic acid</td>
<td>25.0</td>
</tr>
<tr>
<td>2-MB</td>
<td>20.8</td>
</tr>
<tr>
<td>des MB-mevinolinic acid/</td>
<td>1.2:1</td>
</tr>
<tr>
<td>2-MB</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> See legend to Fig. 4 for incubation conditions.  
<sup>b</sup> Abbreviations: des MB-mevinolinic acid, des-(2)-S-methylbutyl mevinolinic acid; 2-MB, 2-methylbutyrate.

synthesis of mevinolinic acid, which was highest at day 3, the acetate incorporation into fatty acids and sterols (ergosterol) was maximum on day 1 of growth and then decreased to virtually zero by days 2 and 3. About 70% of the mevinolinic acid was found to be intracellular, with the remainder in the media. It should also be noted that medium glucose was exhausted, and cell weight had reached a plateau by day 2 of growth (data not shown).

The activities of two key enzymes involved in the biosynthesis of fatty acids were also examined. The pattern of activities of fatty acid synthetase and citrate cleavage enzyme paralleled that of the incorporation of acetate into fatty acids, with maximum activity on day 1 of growth and minimal activity by day 3 (Fig. 3).

The incorporation of other substrates into mevinolinic acid was also studied. Of all the possible substrates (glucose, citrate, oleate, glycerol, propionate, butyrate, and β-hydroxybutyrate) only [methyl-14C]methionine was incorporated at a rate comparable to that of acetate. The incorporation of [methyl-14C] from methionine was linear for at least 30 min.

Identification of the hydrolysis products of mevinolinic acid.

It was important to determine whether the rapid incorporation of radioactive acetate and methionine into mevinolinic acid was due to the de novo synthesis of each portion of the molecule at approximately the same rates or simply represented the incorporation into one portion followed by esterification to a pre-existing mole of the other. The isolated radioactive mevinolinic acid was hydrolyzed, and the alcohol and acid portions were examined for the incorporation of radioactive methionine and acetate by separation on silica gel G plates.

Thin-layer chromatography of the hydrolyzed mevinolinic acid is shown in Fig. 4. Three radioactive peaks were seen, corresponding to des-(2)-S-methylbutyl mevinolinic acid (RF = 0.25), the unhydrolyzed mevinolinic acid (RF = 0.50), and 2-methylbutyric acid (RF = 0.80). A summary of the incorporation of radioactivity into the two parts of the molecule is presented in Table 1. The ratio of [methyl-14C]methionine incorporation into des-(2)-S-methylbutyl mevinolinic acid and into 2-methylbutyrate was 1.2:1, and the ratio for the incorporation of [1-14C]acetate was 7.4:2, which was close to the expected ratio of 9:2 if 9 mol of acetate was incorporated into des-(2)-S-methylbutyl mevinolinic acid and 2 mol was incorporated into 2-methylbutyric acid. Since 2-methylbutyric acid does not show any UV absorption, its identity was confirmed by HPLC of the bromophenacetyl ester derivative.

This experiment demonstrated that a methyl group from methionine was incorporated into the 2-methylbutyric acid side chain as well as into the des-(2)-S-methylbutyl mevinolinic acid at C-8. We also corroborated the work of Chan et al. (4), that 2 mol of acetate is incorporated into the 2-methylbutyrate side chain. Furthermore, these studies indicated that both portions of the molecule were synthesized at approximately the same rate. The mechanism of synthesis of the 2-methylbutyrate most probably involves a "thiolase-like" condensation of 2 mol of acetyl CoA, followed by the addition of methyl from S-adenosylmethionine at the methylene carbon of acetoacetyl CoA.

These studies show that the biosynthesis of mevinolinic acid is inversely related to that of fatty acids in these cells. The similarity of the enzymes for the synthesis of other polyketides (6-methylsalicylic acid) and fatty acid synthase (5) pose the intriguing question of whether this is also true in A. terreus. The activity of fatty acid synthase decreased as mevinolinic acid synthesis increased. Two obvious alternative explanations are suggested: (i) a completely new mevinolinic acid synthase is produced by the organism in response to a signal that growth has ceased; or (ii) the existing fatty acid synthase is modified to produce a C18 polyketide chain instead of a long-chain fatty acid. The decrease in

![Graph](http://jb.asm.org/Downloaded from http://jb.asm.org)
citrate cleavage enzyme also poses a problem in accounting for the availability of acetyl CoA to the cytoplasm for the biosynthesis of mevinolinic acid, assuming that the synthesis of this molecule is extramitochondrial (10).

Other questions on the control of mevinolinic acid biosynthesis, such as how the chemistry of ring closure, formation of the double bonds, reduction of the carbonyl and hydroxyl groups, addition of the methyl to C-6, and formation and esterification of the 2-methylbutyric acid is organized, will only be forthcoming when the putative mevinolinic acid synthase is isolated and characterized.

ACKNOWLEDGMENTS

We thank Alfred W. Alberts for many helpful discussions during this work, Richard Monaghan and Thomas Hallida for help in growing the organism, Henry Joshua for assistance with the HPLC assay for mevinolinic acid, and Joan Kiliyanski for typing this manuscript.

LITERATURE CITED


