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Purification of a 40-Kilodalton Methyltransferase Active in the Aflatoxin Biosynthetic Pathway

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The penultimate step in the aflatoxin biosynthetic pathway of the filamentous fungi *Aspergillus flavus* and *A. parasiticus* involves conversion of sterigmatocystin to *O*-methylsterigmatocystin. An *S*-adenosylmethionine-dependent methyltransferase that catalyzes this reaction was purified to homogeneity (>90%) from 78-h-old mycelia of *A. parasiticus* SRRC 163. Purification of this soluble enzyme was carried out by five soft-gel chromatographic steps: cell debris remover treatment, QMA ACELL chromatography, hydroxylapatite-Ultrogel chromatography, DEAE-Spherodex chromatography, and Octyl Avidgel chromatography, followed by MA7Q high-performance liquid chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the protein peak from this step on silver staining identified a single band of approximately 40 kDa. This purified protein was distinct from the dimeric 168-kDa methyltransferase purified from the same fungal strain under identical growth conditions (D. Bhatnagar, A. H. J. Ullah, and T. E. Cleveland, Prep. Biochem. 18:321–349, 1988). The chromatographic behavior and N-terminal sequence of the 40-kDa enzyme were also distinct from those of the 168-kDa methyltransferase. The molar extinction coefficient of the 40-kDa enzyme at 278 nm was estimated to be 4.7 × 10^4 M^{-1} cm^{-1} in 50 mM potassium phosphate buffer (pH 7.5).

The filamentous fungi *Aspergillus flavus* Link and *A. parasiticus* Speare produce aflatoxins, which are secondary metabolites with carcinogenic and mutagenic properties (23, 26). The ubiquitous presence of these fungi in foods and feeds and their aflatoxin-producing capability have stimulated an interest in understanding the aflatoxin biosynthetic pathway. Previous studies have determined that aflatoxins originate from a polyketide precursor and are proposed to be synthesized in the following manner: polyketide precursor → norsolorinic acid (16) → averantin, (3) → averufanin (24) → averufin (22) → hydroxyversicolorone (31) → versiconal hemiacetal acetate (29) → versicolorin A (21) → sterigmatocystin (ST) (15) → *O*-methylsterigmatocystin OMST (7) → aflatoxin B_1. Recently, versicolorin B has been demonstrated to be a precursor of versicolorin A (25, 33). There is a proposed branch point in the pathway following versiconal hemiacetal acetate production leading to the different aflatoxin structural forms B_1 and B_2 (6, 13, 32, 34).

Specific enzyme activities have been associated with precursor conversions in the aflatoxin pathway (2, 4, 6, 8–12, 17, 33–36). In particular, the conversions of sterigmatocystin and dihydrosterigmatocystin to *O*-methylsterigmatocystin and dihydro-*O*-methylsterigmatocystin, respectively, require a methyltransferase (Fig. 1). In two separate studies, methyltransferases which catalyze these conversions have been identified (10, 35). Bhatnagar et al. (8) purified a 168-kDa methyltransferase to homogeneity from *A. parasiticus* SRRC 163. Yabe et al. (35) identified two distinct methyltransferase activities in cell extracts of *A. parasiticus* NRRL 2999 which migrated with the 180- and 210-kDa fractions on a gel filtration column. The former activity could correspond to the protein purified by Bhatnagar et al. (8) because it catalyzed ST-to-OMST conversion, whereas the 210-kDa fraction methylated only demethylsterigmatocystin to yield ST.

Nearly 40% of the total methyltransferase activity from *A. parasiticus* SRRC 163 cell extracts was a cationic species at pH 7.5, as observed by Bhatnagar et al. (8) during purification of the 168-kDa methyltransferase. This cationic activity catalyzed the same reaction (ST → OMST) as that carried out by the 168-kDa anionic methyltransferase (8). The purification to homogeneity and characterization of this second methyltransferase from *A. parasiticus* SRRC 163 is reported here.

(A preliminary report of this study was presented earlier [19].)

MATERIALS AND METHODS

Fungal strain and culture conditions. *A. parasiticus* SRRC 163, a nitrosoguanidine mutant of aflatoxin-producing strain SRRC 143, was used in this study. SRRC 163 does not produce aflatoxin B_1 but accumulates the precursor averantin (3). The enzymes required for the latter stages of aflatoxin biosynthesis (ST → OMST → aflatoxin B_1) are present in this strain (12).

The fungus was grown on potato dextrose agar plates for 10 days at 29°C in darkness. A 1.0-ml portion of the culture spore suspension (10^8 spores per ml) was transferred to each of 10 2.8-liter Fernbach flasks containing 1 liter of A&M growth medium (1) with sucrose substituted for glucose. Cultures were incubated on a rotary shaker (150 rpm) for 84 h at 29°C, after which fungal mycelia were harvested for preparation of cell extracts.

Cell extracts. Routinely, mycelia (200 to 250 g [wet weight]) were harvested by vacuum filtration and washed extensively with 0.05 M potassium phosphate buffer, pH 7.5.

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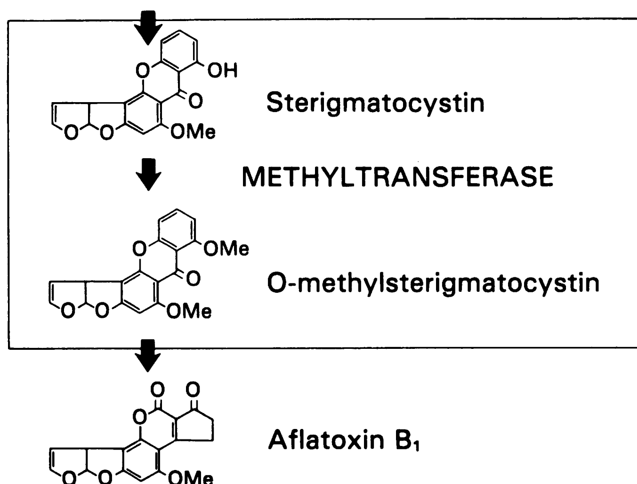

FIG. 1. Schematic representation of the conversion of ST to OMST by a methyltransferase in cell extracts of *A. parasiticus* SRRC 163.

Mycelia frozen under liquid nitrogen were pulverized to a fine powder in the presence of liquid nitrogen in a Waring blender as described previously (21). The powdered mycelia were suspended in the above-described buffer containing 5% glycerol, 2 mM 2-mercaptoethanol, and 100 μM phenylmethylsulfonyl fluoride (buffer A). The resultant mixture was filtered through Miracloth, and cellular debris was removed by centrifugation at 10,000 × *g*. The supernatant was used for purification of the methyltransferase.

General methods. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed on homogeneous 12.5 or 20% PhastGel acrylamide preformed gels by using the PhastSystem (Pharmacia). Low-molecular-weight standards for molecular weight determination of the denatured enzyme were purchased from Bio-Rad Laboratories. Protein concentration was estimated by a Coomassie G-250 dye-binding assay (28) by using ovalbumin as the standard. For excision of protein bands from native gels, proteins were separated on a Protean II xi Vertical Electrophoresis Cell (Bio-Rad) and the gel was negatively stained with Quick-Stain (Diversified Biotech) to retain enzyme activity.

High-performance liquid chromatography (HPLC) was performed by using an ISCO system with model 2350 pumps, a V^4 detector, and Chemresearch software. MA7Q (Bio-Rad) and VYDAC RPC18 columns were used for HPLC separations. N-terminal amino acid sequence determination was performed on a PI2090 on-line gas-phase sequencer (Porton Instruments).

Methyltransferase assay. An aliquot of cell extract from each purification step was added to a 1-ml final volume of buffer A containing 500 μM *S*-adenosylmethionine (SAM) (Sigma). Reactions were initiated by adding 50 μmol of ST in 10 μl of acetone, and the mixture was incubated for 30 min at 25°C with constant agitation. The assay was terminated and the reaction products were extracted by addition of 4 ml of chloroform. The metabolites were separated and identified by one-dimensional silica gel thin-layer chromatography (TLC) (20 by 20 cm; Merck & Co.) in ether-methanol-water (96:3:1) in which the substrate, ST (R_f, 0.97), and product, OMST (R_f, 0.44), were distinctly separated. TLC plates were scanned densitometrically for fluorescent material on a CS-930 dual-wavelength TLC scanner (Shimadzu, Kyoto, Japan); the ST and OMST concentrations (excitation wavelength, 310 nm) were quantitated by comparison with concentrations of known standards spotted on the same plate. One unit of enzyme activity refers to 1 pmol of OMST produced per s (1 pkat).

Protein characteristics. The effects of pH, temperature, time of incubation, and enzyme concentration on methyltransferase activity were determined on protein samples from step 6 (see Results and Table 1). The methyltransferase assay was modified as follows: (i) to assess the effect of pH on enzyme activity, the pH of the assay medium was adjusted by using 50 mM glycine-HCl for pH 2.0 to 3.0, 50 mM sodium acetate for pH 4.0 to 5.0, 50 mM potassium phosphate for pH 6.0 to 7.5, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) for pH 8.0 to 8.5, and 50 mM Tris-acetate for pH 9.4; (ii) to assess the effect of temperature on enzyme activity, the enzyme assays were performed at a temperature range of 10 to 60°C; (iii) to assess the effect of time of incubation on enzyme activity, the enzyme assays were incubated from 15 min to 2 h; and (iv) to assess the effect of enzyme concentration on enzyme activity, the enzyme concentration was varied from 0.01 to 50 μg/ml. These assays were compared with standard assay conditions in which the reaction was carried out at pH 7.5 in buffer A with constant shaking at ca. 25°C for 30 min with an enzyme concentration of 15 μg/ml. The Michaelis-Menten constant, apparent K_m values, for ST and SAM were determined by Lineweaver-Burk plots, and the data were analyzed by linear regression. The molar extinction coefficient of methyltransferase was estimated on the basis of the molecular mass and optical absorbance (278 nm) of the protein in 12.5 mM potassium phosphate buffer, pH 7.5, containing 150 mM KCl. Both the enzyme kinetics and optical properties of the methyltransferase were determined from protein samples obtained from step 6; the subunit molecular weight of the protein was determined by comparison with Bio-Rad low-molecular-weight standards after SDS-PAGE.

RESULTS

Protein purification. All soft-gel steps were done at 4°C, and HPLC steps were done at room temperature.

Step 1. CDR chromatography. The 10,000 × *g* supernatant of the cell extract (step 0) was mixed with cell debris remover (CDR; Whatman) at 15 g of CDR per 100 ml of supernatant. The suspension was stirred for 60 min and filtered through Miracloth, and the filtrate was collected. CDR is a weak anion-exchange medium, and approximately 5% of the total methyltransferase activity bound to the CDR matrix.

Step 2. QMA ACELL chromatography. The filtrate was applied to a QMA ACELL (Waters) anion-exchange column (2.5 by 20 cm), previously equilibrated with buffer A, at a flow rate of 100 ml/h. Methyltransferase activity retained on the column represented the previously described anionic species (8). The methyltransferase activity in the effluent represented the cationic species, the enzyme described in this communication.

Step 3. Hydroxylapatite-Ultrogel chromatography. Protein in the effluent was fractionated by precipitation with ammonium sulfate (0 to 35, 35 to 65, and >65% saturation). The methyltransferase activity was observed in the 35 to 65% saturation fraction; the precipitated protein was collected by centrifugation at 10,000 × *g* for 30 min. The pellet was dissolved in buffer A and extensively dialyzed against 5 mM

potassium phosphate buffer, pH 7.5, with 5% glycerol and 2 mM 2-mercaptoethanol. The dialyzed fraction was then applied to a hydroxylapatite-Ultrogel (Bio-Rad) column (2.5 by 12 cm), previously equilibrated in 5 mM potassium phosphate buffer, at a flow rate of 50 ml/h. The cationic methyltransferase activity did not bind to this column and was eluted entirely in the effluent; binding of proteins to this column is based on nonspecific electrostatic interactions.

Step 4. DEAE-Spherodex column chromatography. The effluent was adjusted to 12.5 mM potassium phosphate (pH 7.5) with 1.0 M potassium phosphate (pH 7.5) and applied (50 ml/h) to a DEAE-Spherodex (IBF Biotechnics) column (2.5 by 12 cm) previously equilibrated with 12.5 mM potassium phosphate buffer. Proteins were eluted with a linear gradient of 0 to 0.3 M KCl in the column buffer (140 ml) at a flow rate of 50 ml/h followed by 30 ml of 0.5 M KCl in the same buffer. The fractions (4 ml) were assayed for methyltransferase activity; peak activity was observed at 0.175 M KCl.

Step 5. Octyl Avidgel chromatography. The active fractions from step 4 were pooled, adjusted to 2.0 M ammonium sulfate, and applied to an Octyl Avidgel (BioProbe International, Inc.) column (2.5 by 3 cm), previously equilibrated in 12.5 mM potassium phosphate buffer containing 2.0 M ammonium sulfate, at a flow rate of 75 ml/h. Protein fractions were eluted in steps of 1.8, 1.5, 1.35, 1.2, and 0 M ammonium sulfate, depending on the extent of hydrophobic interaction of the proteins and the matrix. The most active fraction was eluted at 1.35 M ammonium sulfate.

Step 6. Anion-exchange HPLC. The methyltransferase activity from the previous step was precipitated by ammonium sulfate at 70% saturation. The pellet was suspended in 15 mM HEPES buffer, pH 8.0, with 5% glycerol and 2 mM 2-mercaptoethanol and dialyzed in the same buffer to remove traces of ammonium sulfate. Aliquots of the preparation were applied to one of two MA7Q columns (50 by 7.8 mm analytical or 100 by 19 mm preparative; Bio-Rad) and eluted by a linear gradient of 0 to 0.3 M NaCl in 25 mM HEPES buffer, pH 8.5, containing 5% glycerol and 2 mM 2-mercaptoethanol at a flow rate of 30 ml/h. The two MA7Q columns were used to obtain purified protein fractions for different analyses. The analytical MA7Q column resulted in >90% enzyme purity as determined by densitometric analysis following SDS-PAGE. This preparation was used to determine protein characteristics and to clean up the protein sample by reversed-phase HPLC for sequencing. Higher yields of the protein were required for confirmation of the identity of the methyltransferase by excising the protein from a nondenaturing gel and determining activity; the preparative MA7Q column was used for this purpose. This preparative column resulted in lower protein purity (<75%; Fig. 2) than the smaller MA7Q column (>90%; Fig. 2), but the former column was more expedient for the purpose described. The active fraction eluted from both MA7Q columns between 0.135 and 0.150 M NaCl.

Purity. The methyltransferase was purified approximately 90-fold to a specific activity of 352 pkat/mg after one run on the analytical MA7Q column, step 6 (Table 1). As previously described (8), part of the methyltransferase activity in steps 0 and 1 (the 10,000 × g supernatant and CDR chromatography, respectively) was attributable to the presence of the 168-kDa methyltransferase in these fractions. The two methyltransferases were separated in step 2 (QMA ACELL chromatography). Step 5 (Octyl Avidgel chromatography) resulted in a loss of yield with no significant gain in fold purification, but this step was included because it did remove some proteins that otherwise were difficult to separate from the methyltransferase in the next step (anion-exchange HPLC; MA7Q). An additional step, reversed-phase HPLC, was included to clean up the >90% methyltransferase from the analytical MA7Q column for N-terminal sequencing. The protein obtained from this procedure was not used for activity studies because the enzyme was rendered inactive under the conditions used. The single protein that eluted from the C18 column was estimated to be the methyltrans-

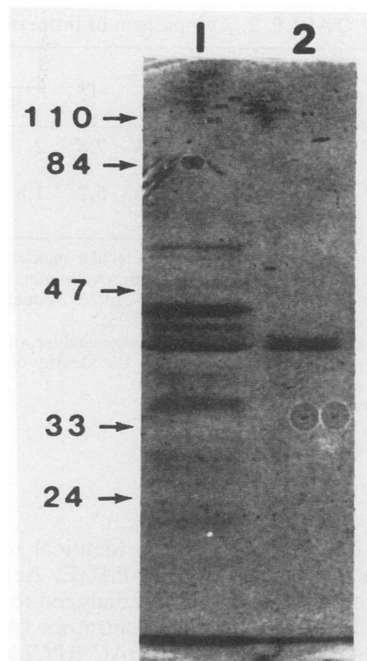

FIG. 2. SDS-polyacrylamide gel stained with Coomassie blue. Molecular masses in kilodaltons are indicated on the left. Individual protein bands were excised from a nondenaturing gel of the methyltransferase active fraction after anion exchange on a preparative (100 by 19 mm) MA7Q HPLC column (this fraction is shown in lane 1). Only one band (lane 2) showed methyltransferase activity; this protein was estimated to be 40 kDa when electrophoresed on SDS-polyacrylamide gels.

TABLE 1. Purification of 40-kDa methyltransferase

Step[a]	Vol (ml)	Total enzyme activity (pkat)	Total protein (mg)	Sp act[b]	Fold purification	Yield (%)
0	1,800	11,880	3,018	3.9	1	100
1	1,825	12,045	1,965	6.0	1.5	101.4
2	1,700	10,540	1,286	8.2	2.1	88.7
3	332	10,956	260	42.0	10.8	92.2
4	131	7,205	139.5	52.0	13.3	60.6
5	300	2,520	49.1	51.0	13.1	21.2
6[c]	0.8	35.2	0.1	352	90.3	ND[d]

[a] Steps: 0, 10,000 × g supernatant of cell extracts from a mycelial wet weight of 200 g; 1, CDR; 2, QMA ACELL anion exchange; 3, hydroxylapatite-Ultrogel; 4, DEAE-Spherodex; 5, Octyl Avidgel; 6, Anion-exchange HPLC (MA7Q column).

[b] Expressed in picokatals per milligram of protein at 25°C, where 1 pkat = 1 pmol of OMST produced per s.

[c] Data for step 6 represent one run on the HPLC MA7Q column (50 by 7.8 mm); each preparation would provide material for approximately 20 runs.

[d] ND, not determined.

TABLE 2. Comparison of properties of two methyltransferases purified from *A. parasiticus* SRRC 163

Methyl-transferase	No. of subunits	Optimum temp (°C)	Optimum pH	pI	K_m (μM), r^2		Turnover no. (10^{-2} s^{-1})	Molar extinction coefficient (278 nm; 10^4 M^{-1} s^{-1})	N terminus
					ST	SAM			
40 kDa	1[a]	40–45	7–9.4	7.8[b]	2, 0.82[c]	9.6, 0.74[c]	1.4	4.7	S-E-R-T-P-S-D-N-E-H-A-Q-A-X[d]-E-I-V-R-T-Q-D-P
168 kDa[e]	2[f]	25–35	7.5–8.0	5.2	1.8	42	2.2	7.87	S-P-L-V-A-T-D-F-T-L-A-V[g], N-L-L-K-D-P/L-K-G-V-P-H-P-D[h]

[a] Estimated to be monomeric since the molecular masses determined by size exclusion chromatography (column calibrated with *Saccharomyces cerevisiae* alcohol dehydrogenase, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor) and denaturing SDS-PAGE were identical. The protein also migrated very close to a 38-kDa protein (reductase, reference 4) on gel filtration chromatography.

[b] Estimated by chromatofocusing (reference 5).

[c] Correlation coefficient for a linear regression analysis of 1/S and 1/V.

[d] Residue 14 is reported as unknown since the identity of the amino acid is uncertain.

[e] Reference 8.

[f] 110 and 58 kDa.

[g] 110-kDa subunit.

[h] 58-kDa subunit.

ferase, since it exhibited mobility identical to that of the active protein from step 6 in SDS-PAGE. Active fractions from step 6 were concentrated and dialyzed to 0.1% trifluoroacetic acid by Centriprep Concentrators (Amicon). The sample was then applied to a VYDAC RPC18 column (250 by 4.6 mm) and eluted with a linear gradient of 0 to 80% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 30 ml/h. The protein was eluted as a single component at ca. 55% acetonitrile.

The identity of the methyltransferase was determined by excising individual protein bands from a nondenaturing gel loaded with the active fraction from the preparative MA7Q HPLC column, step 6 (Fig. 2). Only one band catalyzed the conversion of ST to OMST; when subjected to SDS-PAGE, this band extracted from the nondenaturing gel revealed a single 40-kDa protein (Fig. 2). This band (identified by silver staining) coincided with the single protein band eluted from the RPC18 column and with the major band (>90%) from the analytical MA7Q column. The purity of this material was also ascertained upon stepwise Edman degradation of the 40-kDa band on a Porton PI2090 sequencer, which provided a single N-terminal sequence through residue 22 (Table 2).

Enzyme activity and optical properties. The temperature optimum under the conditions described in Materials and Methods was between 40 and 45°C; the enzyme exhibited only 35% of its activity at 25°C (data not shown) and retained ca. 30% of its activity at 60°C (data not shown). The enzyme was equally active between pHs 7.0 and 9.4 (data not shown). The methyltransferase activity was found to be linear (data not shown) with respect to enzyme concentration (0.01 to 50 μg of purified protein) when assayed for 30 min (see Materials and Methods), and the activity was also linear with incubation time between 15 min and 2 h at 15 μg of purified protein in a 1-ml assay mixture.

The K_ms for both substrates, ST and SAM, were determined by varying their concentrations in reaction mixtures with 15 μg of the purified enzyme (1.0 to 25 μM ST and 5 to 400 μM SAM). The apparent K_m for ST was 2.0 μM, r^2 = 0.82 (at 500 μM SAM), and that for SAM was 9.6 μM, r^2 = 0.74 (at 50 μM ST) (Table 2). Considering the complex mechanics of the methyltransferase assay (TLC followed by scanning densitometry) and the limitation in substrate solubility in aqueous medium, the correlation coefficients (r^2) indicated a reasonable fit to the Michaelis-Menton model for enzyme kinetics. The turnover number of the enzyme was calculated to be 1.4×10^{-2}/s (Table 2) on the basis of a specific activity of 350 pmol of OMST produced per s per mg of protein (molecular mass, 40 kDa).

The UV and visible spectra of the purified enzyme (from step 6) in 12.5 mM potassium phosphate (pH 7.5)–150 mM KCl showed maximum absorbance at 278.8 nm; no absorption was observed in the visible region. On the basis of a molecular mass of 40 kDa, the molar extinction coefficient was estimated to be 4.7×10^7 M^{-1} cm^{-1} (Table 2). A comparison of properties of the previously purified 168-kDa methyltransferase (8) and the 40-kDa methyltransferase described in this communication is presented in Table 2.

DISCUSSION

Purification of a 40-kDa methyltransferase from *A. parasiticus* SRRC 163 has resulted in the characterization of a second enzyme from this system which converts ST to OMST in vitro (Fig. 1). The existence of this enzyme was observed when different chromatographic properties allowed separation of the two enzymes: the 168-kDa methyltransferase was anionic at pH 7.5 and bound to QMA ACELL resin, whereas the 40-kDa methyltransferase was cationic at this pH and did not bind to QMA. When characteristics of the 40-kDa methyltransferase are compared with those of the previously purified 168-kDa enzyme (8), several distinct differences between the two in terms of molecular mass and N-terminal sequences are observed (Table 2). Both proteins appear to be minor constituents in the *A. parasiticus* mycelia, as indicated by the degree of purification required to achieve homogeneity; over 160-fold for the 168-kDa methyltransferase (8) and 90-fold for the 40-kDa methyltransferase at the final purification step (step 6; Table 1). Some difficulties in purifying enzymes involved in secondary metabolic pathways are that they are often present in relatively low concentrations and are extremely short-lived (8, 14).

It is unknown whether the two methyltransferases are related to each other. Although preliminary sequencing data indicate that their N termini differ, this does not rule out the possibility that the 40-kDa protein has some sequence homology to the 168-kDa protein. Antibodies raised against a preparation of the larger protein (12) reacted weakly with the 40-kDa protein (data not shown); antibodies against the 40-kDa protein need to be tested with the 168-kDa protein. Several of the kinetic and optical properties of the two methyltransferases were similar (Table 2). As described

previously, these properties of the 40-kDa protein were determined on samples of the methyltransferase from the analytical MA7Q HPLC column (step 6). The values of the correlation coefficients obtained by analysis of the kinetic data (Table 2) can be accounted for by the nature of the methyltransferase assay, which included extraction of compounds from the aqueous phase, separation of components by TLC, and quantitation by scanning densitometry. As replications for the experiments yielded consistent figures, the results presented here are characteristic of the 40-kDa methyltransferase.

It was observed that the ratio of total methyltransferase activity attributed to the 40- and 168-kDa proteins varied, sometimes depending on mycelial age and growth conditions, but not in any consistent manner; this was in agreement with previous studies (5). Several methyltransferase activities pertaining to aflatoxin biosynthesis have been identified; three of them catalyze the reaction of ST to OMST (this report; 8, 35), and one catalyzes a step proposed to immediately precede the conversion of ST to OMST (35). The relationship between these different methyltransferases and their participation in aflatoxin biosynthesis will become clearer in future purification studies aimed at (i) further characterization and sequencing of the enzymes, (ii) identification and sequencing of the genes that encode these enzymes, and (iii) site-directed mutagenesis and/or gene disruption. Candidate sites for mutagenesis experiments would include any conserved domains in these methyltransferases. One characteristic of other methyltransferases has been the appearance of conserved amino acid motifs (18, 20, 27, 30). The consensus sequences are most notable among the DNA methyltransferases (20, 27), but there appears to be a conserved SAM-binding site of three specific amino acids in almost all methyltransferases (18, 30). The identification and manipulation of these and other conserved motifs in genes that encode the 168- and 40-kDa methyltransferases may assist in determining the genetic origins and functions of these two enzymes in aflatoxin biosynthesis. These studies are currently being done in our laboratory.

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REFERENCES

1. **Adye, J., and R. I. Mateles.** 1964. Incorporation of labelled compounds into aflatoxins. Biochim. Biophys. Acta **86:**418–420.
2. **Anderson, J. A., C. H. Chung, and S.-H. Cho.** 1990. Versicolorin A hemiacetal, hydroxydihydrosterigmatocystin, and aflatoxin G_{2a} reductase activity in extracts from *Aspergillus parasiticus*. Mycopathologia **111:**39–45.
3. **Bennett, J. W., L. S. Lee, S. M. Shoss, and G. H. Boudreaux.** 1980. Identification of averantin as an aflatoxin B_1 precursor. Appl. Environ. Microbiol. **39:**835–839.
4. **Bhatnagar, D., and T. E. Cleveland.** 1990. Purification and characterization of a reductase from *Aspergillus parasiticus* SRRC 2043 involved in aflatoxin biosynthesis. FASEB J. **4:**2727.
5. **Bhatnagar, D., and T. E. Cleveland.** 1991. Aflatoxin biosynthesis: developments in chemistry, biochemistry, and genetics, p. 391–405. *In* O. L. Shotwell and C. R. Hurburg, Jr. (ed.), Aflatoxin in corn—new perspectives. Iowa State University, Ames.
6. **Bhatnagar, D., T. E. Cleveland, and D. G. I. Kingston.** 1991. Enzymological evidence for separate pathways for aflatoxin B_1 and B_2 biosynthesis. Biochemistry **30:**4343–4350.
7. **Bhatnagar, D., S. P. McCormick, L. S. Lee, and R. A. Hill.** 1987. Identification of *O*-methylsterigmatocystin as an aflatoxin B_1 and G_1 precursor in *Aspergillus parasiticus*. Appl. Environ. Microbiol. **53:**1028–1033.
8. **Bhatnagar, D., A. H. J. Ullah, and T. E. Cleveland.** 1988. Purification and characterization of a methyltransferase from *Aspergillus parasiticus* SRRC 163 involved in aflatoxin biosynthetic pathway. Prep. Biochem. **18:**321–349.
9. **Chuturgoon, A. A., M. F. Dutton, and R. K. Berry.** 1990. The preparation of an enzyme associated with aflatoxin biosynthesis by affinity chromatography. Biochem. Biophys. Res. Commun. **166:**38–42.
10. **Cleveland, T. E., and D. Bhatnagar.** 1987. Individual reaction requirements of two enzyme activities, isolated from *Aspergillus parasiticus*, which together catalyze conversion of sterigmatocystin to aflatoxin B_1. Can. J. Microbiol. **33:**1108–1112.
11. **Cleveland, T. E., and D. Bhatnagar.** 1990. Evidence for *de novo* synthesis of an aflatoxin pathway methyltransferase near the cessation of active growth and the onset of aflatoxin biosynthesis by *Aspergillus flavus* mycelia. Can. J. Microbiol. **36:**1–5.
12. **Cleveland, T. E., D. Bhatnagar, C. J. Foell, and S. P. McCormick.** 1987. Conversion of a new metabolite to aflatoxin B_2 by *Aspergillus parasiticus*. Appl. Environ. Microbiol. **53:**2804–2807.
13. **Cleveland, T. E., A. R. Lax, L. S. Lee, and D. Bhatnagar.** 1987. Appearance of enzyme activities catalyzing conversion of sterigmatocystin to aflatoxin B_1 in late-growth-phase *Aspergillus parasiticus* cultures. Appl. Environ. Microbiol. **53:**1711–1713.
14. **Dutton, M. F.** 1988. Enzymes and aflatoxin biosynthesis. Microbiol. Rev. **52:**274–295.
15. **Hsieh, D. P. H., M. T. Lin, and R. T. Yao.** 1973. Conversion of sterigmatocystin to aflatoxin B_1 by *Aspergillus parasiticus*. Biochem. Biophys. Res. Commun. **52:**992–997.
16. **Hsieh, D. P. H., M. T. Lin, R. C. Yao, and R. Singh.** 1976. Biogenesis of aflatoxins: conversion of norsolorinic acid and other hypothetical intermediates into aflatoxin B_1 by *Aspergillus parasiticus*. J. Agric. Food Chem. **24:**1167–1170.
17. **Hsieh, D. P. H., C. C. Wan, and J. A. Billington.** 1989. A versiconal hemiacetal acetate converting enzyme in aflatoxin biosynthesis. Mycopathologia **107:**121–126.
18. **Ingrosso, D., A. V. Fowler, J. Bleibaum, and S. Clarke.** 1989. Sequence of the D-aspartyl/L-isoaspartyl protein methyltransferase from human erythrocytes. J. Biol. Chem. **264:**20131–20139.
19. **Keller, N. P., H. C. Dischinger, Jr., D. Bhatnagar, T. E. Cleveland, and A. H. J. Ullah.** 1991. Purification of second methyltransferase active in the aflatoxin biosynthetic pathway. FASEB J. **5:**2623.
20. **Lauster, R.** 1989. Evolution of type II DNA methyltransferases, a gene duplication model. J. Mol. Biol. **206:**313–321.
21. **Lee, L. S., J. W. Bennett, A. F. Cucullu, and R. L. Ory.** 1976. Biosynthesis of aflatoxin B_1 conversion of versicolorin A to aflatoxin B_1 by *Aspergillus parasiticus*. J. Agric. Food Chem. **24:**1167–1170.
22. **Lin, M. T., D. P. H. Hsieh, R. C. Yao, and J. A. Donkersloot.** 1973. Conversion of averufin into aflatoxins by *Aspergillus parasiticus*. Biochemistry **12:**5167–5171.
23. **Martin, C. N., and R. C. Garner.** 1977. Aflatoxin B_1-oxide generated by chemical or enzymatic oxidation of aflatoxin B_1 causes guanine substitution in nucleic acids. Nature (London) **267:**863–865.
24. **McCormick, S. P., D. Bhatnagar, and L. S. Lee.** 1987. Averufanin is an aflatoxin B_1 precursor between averantin and averufin in the biosynthetic pathway. Appl. Environ. Microbiol. **53:**14–16.
25. **McGuire, G. M., S. W. Brobst, T. L. Graybill, K. Pal, and C. A. Townsend.** 1989. Partitioning of tetrahydro- and dihydrobisfuran formation in aflatoxin biosynthesis defined by cell-free and direct incorporation experiments. J. Am. Chem. Soc. **111:**8308–8309.
26. **McMahon, G., E. F. Davis, L.-J. Huber, Y. Kim, and G. N. Wogan.** 1986. Characterization of c-Ki-*ras* and N-*ras* in aflatoxin B_1-induced rat liver tumors. Proc. Natl. Acad. Sci. USA **83:**9418–9422.
27. **Posfai, J., A. S. Bhagwat, G. Posfai, and R. Roberts.** 1989.

Predictive motifs derived from cytosine methyltransferases. Nucleic Acids Res. **17**:2421–2435.

28. **Sedmak, J. J., and S. E. Grossberg.** 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. **79**:544–552.
29. **Shroeder, H. W., R. J. Cole, R. D. Grigsby, and H. Hein, Jr.** 1974. Inhibition of aflatoxin production and tentative identification of an aflatoxin intermediate "versiconal acetate" from treatment with dichlorvos. Appl. Microbiol. **27**:394–399.
30. **Smith, H. O., T. Annua, and S. Chandrasegaran.** 1990. Finding sequence motifs in groups of functionally related proteins. Proc. Natl. Acad. Sci. USA **87**:826–830.
31. **Townsend, C. A., K. A. Plaucen, K. Pal, and S. W. Brobst.** 1988. Hydroxyversicolorone: isolation and characterization of a potential intermediate in aflatoxin biosynthesis. J. Org. Chem. **53**:2472–2477.
32. **Yabe, K., Y. Ando, and T. Hamasaki.** 1988. Biosynthetic relationship among aflatoxins B_1, B_2, G_1, and G_2. Appl. Environ. Microbiol. **54**:2101–2106.
33. **Yabe, K., Y. Ando, and T. Hamasaki.** 1991. Desaturase activity in the branching step between aflatoxins B_1 and G_1 and aflatoxins B_2 and G_2. Agric. Biol. Chem. **55**:1907–1911.
34. **Yabe, K., Y. Ando, and T. Hamasaki.** 1991. A metabolic grid among versiconal hemiacetal acetate, versiconol acetate, versiconol and versiconal during aflatoxin biosynthesis. J. Gen. Microbiol. **137**:2469–2475.
35. **Yabe, K., Y. Ando, J. Hashimoto, and T. Hamasaki.** 1989. Two distinct *O*-methyltransferases in aflatoxin biosynthesis. Appl. Environ. Microbiol. **55**:2172–2177.
36. **Yabe, K., Y. Nakamura, H. Nakajima, Y. Ando, and T. Hamasaki.** 1991. Enzymatic conversion of norsolorinic acid to averufin in aflatoxin biosynthesis. Appl. Environ. Microbiol. **57**:1340–1345.