APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1989, p. 2172–2177
0099-2240/89/092172-06$02.00/0

Vol. 55, No. 9

Two Distinct *O*-Methyltransferases in Aflatoxin Biosynthesis

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Received 27 March 1989/Accepted 31 May 1989

The substances belonging to the sterigmatocystin group bear a close structural relationship to aflatoxins. When demethylsterigmatocystin (DMST) was fed to *Aspergillus parasiticus* NIAH-26, which endogenously produces neither aflatoxins nor precursors in YES medium, aflatoxins B_1 and G_1 were produced. When dihydrodemethylsterigmatocystin (DHDMST) was fed to this mutant, aflatoxins B_2 and G_2 were produced. Results of the cell-free experiment with *S*-adenosyl-[*methyl*-^{3}H]methionine showed that first the C-6-OH groups of DMST and DHDMST are methylated to produce sterigmatocystin and dihydrosterigmatocystin (*O*-methyltransferase I) and then the C-7-OH groups are methylated to produce *O*-methylsterigmatocystin (OMST) and dihydro-*O*-methylsterigmatocystin (DHOMST) (*O*-methyltransferase II). However, no methyltransferase activity was observed when either OMST, DHOMST, 5,6-dimethoxysterigmatocystin, 5-methoxysterigmatocystin, or sterigmatin was incubated with the cell extract. Treatment of the cell extract with *N*-ethylmaleimide inhibited *O*-methyltransferase I activity but not that of *O*-methyltransferase II. Furthermore, these *O*-methyltransferases were different in their protein molecules and were involved in both the reactions from DMST to OMST and DHDMST to DHOMST. The reactions described in this paper were not observed when the same mold had been cultured in YEP medium.

Aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) are major naturally occurring aflatoxins that are produced by certain strains of *Aspergillus flavus* and *A. parasiticus* (2). The biosynthetic pathway of AFB_1 has been extensively studied, and recently it has been reported that aflatoxins (AFB_1 and AFG_1) containing dihydrobisfuran are produced from sterigmatocystin (ST) through the formation of *O*-methylsterigmatocystin (OMST) (3, 15) and that one kind of *O*-methyltransferase and oxidoreductase system is involved in the biosynthetic pathway of AFB_1 (7, 15). Also, our recent studies (15) demonstrated that aflatoxins (AFB_2 and AFG_2) containing tetrahydrobisfuran are independently produced from dihydrosterigmatocystin (DHST) through the formation of dihydro-*O*-methylsterigmatocystin (DHOMST). Moreover, we observed that the same *O*-methyltransferase and oxidoreductase enzymes relating to the formation of AFB_1 are also involved in the biosynthesis of AFB_2.

On the other hand, the STs are a group of closely related fungal metabolites produced by *Aspergillus* spp. and *Bipolaris* spp., and various STs aside from the four precursors described above have been identified. Among them, demethylsterigmatocystin (DMST) (9, 10), dihydrodemethylsterigmatocystin (DHDMST) (12), 5-methoxysterigmatocystin (5-methoxy-ST) (13), and sterigmatin (10) have been isolated from *A. versicolor* (Vuillemin) Tiraboschi, and 5,6-dimethoxysterigmatocystin (dimethoxy-ST) has been isolated from *A. multicolor* (11).

In the present study, we attempted to analyze the steps preceding the formation of ST and DHST by carrying out feeding and cell-free experiments with various substrates. These studies demonstrated that DMST and DHDMST are precursors of aflatoxins and that two distinct *O*-methyltransferases are involved in aflatoxin biosynthesis.

MATERIALS AND METHODS

Microorganisms. The strain used in this study was NIAH-26, a UV-irradiated mutant of aflatoxin-producing *A. parasiticus* strain SYS-4 (NRRL 2999). NIAH-26 produces neither aflatoxins nor precursors (16).

Standard samples of metabolites. The structures of the substances used in this study are shown in Fig. 1. ST, OMST, DHST, and DHOMST were prepared as described before (15). DMST (10) and DHDMST (12) were isolated from mycelia of *A. versicolor* (Vuillemin) Tiraboschi. 5-Methoxy-ST and sterigmatin were also isolated by extraction of the mycelia of *A. versicolor* (Vuillemin) Tiraboschi and column chromatography on silica gel (10). Dimethoxy-ST was isolated from mycelia of *A. multicolor* (11). The concentration of the metabolites in methanol was determined from UV absorption spectra by using molar absorption coefficients as follows: DMST (335 nm), 19,100 (S. Hara, personal communication); DHDMST (335 nm), 19,400 (8); ST (329 nm), 13,100 (8); DHST (325 nm), 16,600 (8); OMST (310 nm), 16,500 (8); DHOMST (311 nm), 17,300 (8); 5-methoxy-ST (331 nm), 12,100 (8); dimethoxy-ST (330 nm), 19,200 (8), sterigmatin (324 nm), 16,900 (10).

A standard kit (Makor Chemicals Ltd., Jerusalem, Israel) was used for the analysis of AFB_1, AFB_2, AFG_1, and AFG_2.

Feeding experiments. The tip culture method (16), in which the Pipetman tip (1 ml; Gilson Medical, Middleton, Wis.) was used as the culture vessel, was used for the feeding experiments. A spore suspension was inoculated into YES medium (2% yeast extract, 20% sucrose) containing each of the STs, and the resultant products were examined. When specified, YEP medium (containing 20% peptone instead of sucrose) was used.

Fluorescence photographs were taken by using a Funa-UV-light (type SL-800F), a Shott 470 KV filter, and TMY 5053 film (Eastman Kodak Co., Rochester, N.Y.).

Methyltransferase assay. The postmitochondrial fraction was prepared from the mutant NIAH-26 as the cell extract (15). The mycelia were ground in a mortar and pestle in a solution containing 0.1 M potassium phosphate buffer (pH 7.5) and 0.2 mg of phenylmethylsulfonyl fluoride per ml. The homogenate was centrifuged at 900 × *g* for 2 min and then at 20,000 × *g* for 15 min, and after the addition of glycerol (final

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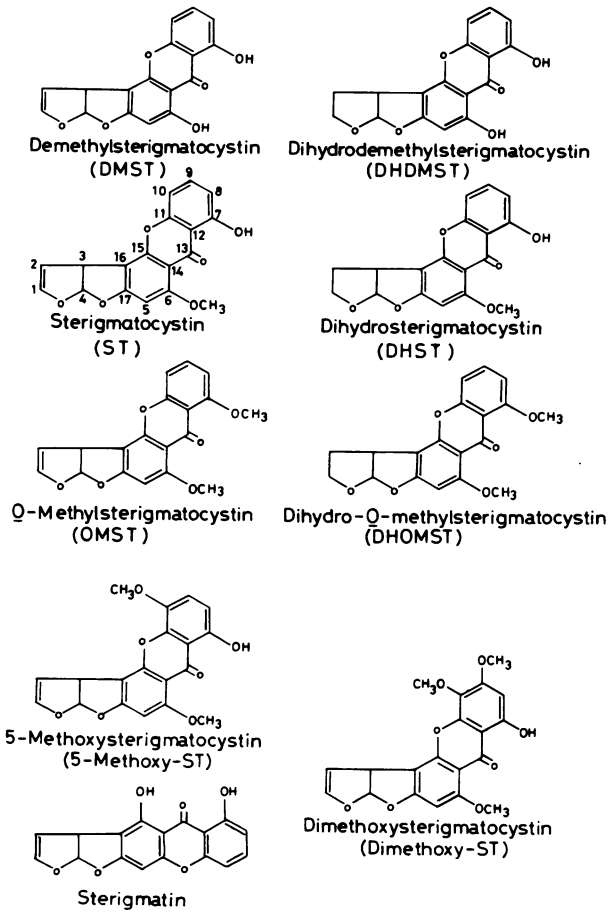

FIG. 1. Structures of the substances used in this study.

concentration, 10%), the 20,000 × *g* supernatant was used for enzyme assays. When specified, the postmicrosomal fraction was prepared by further centrifugation of the cell extract as described below.

The enzyme assay with nonradiolabeled cofactors was also carried out as described before (15).

Methyltransferase activity was detected by using *S*-adenosyl-[*methyl*-^{3}H]methionine ([^{3}H]SAM). The assay was carried out at 37°C in 50 μl of a reaction mixture containing 40 mM phosphate-buffer (pH 7.5), 10% glycerol, 10 μM [^{3}H]SAM (100 μCi/μmol; Amersham Corp., Arlington Heights, Ill.), various STs at 50 μM, and cell extract (0.2 mg of protein per ml). The reaction was started by adding the cell extract and stopped by adding 80 μl of water-saturated chloroform and mixing. The resultant chloroform extract (50 μl) was transferred to a scintillation vial and then mixed with 3 ml of scintillation fluid (Scintisol EX-H; Dojin, Kumamoto, Japan). The radioactivity of the tritium transferred to the chloroform layer was measured in a liquid scintillation spectrometer.

The products of the methylation reactions were determined by thin-layer chromatography (TLC) analyses. After termination of the reaction, 1 μl of the solution containing 2.5 mM ST and 2.5 mM OMST was added to the resultant chloroform layer as an internal marker. Then, 50 μl of the chloroform layer was developed by using TLC-plastic silica gel sheets (no. 5748; Merck & Co., Inc., Rahway, N.J.) with a developing solution composed of chloroform–ethyl acetate–90% formic acid (6:3:1, vol/vol/vol). After examination of the TLC chromatogram under UV light, the site corresponding to ST or OMST was cut out with a pair of scissors. The resultant plastic fragment was immersed into 0.5 ml of ethyl acetate in a vial for 10 min, and then the radioactivity was measured in a liquid scintillation spectrometer with 3 ml of Scintisol EX-H.

The methylation activity was expressed as the total amount of ^{3}H-methylated compounds contained in the chloroform layer.

Treatment of cell extract with NEM. The cell extract (2.4 mg of protein per ml) was incubated with 9.2 mM *N*-ethylmaleimide (NEM) at 37°C for 30 min in a mixture containing 40 mM phosphate buffer (pH 7.5) and 10% glycerol. The reaction was started by the addition of NEM and then stopped by the addition of one-fifth volume of 2 M 2-mercaptoethanol. The methylation activity of NEM-treated enzyme was examined as described above.

Gel filtration. All the methylation activities described in this paper were detected in the postmicrosomal fraction of the mycelia. The postmicrosomal fraction was prepared by centrifugation of the cell extract at 105,000 × *g* for 90 min and was discharged through a column guard (SJHV 004; Millipore Corp., Bedford, Mass.) to remove floating substances. The resultant solution was applied to a combined gel filtration column (Asahipak GS-520P and GS-320P; Asahikasei, Kawasaki, Japan) fitted to the Shimadzu 6A chromatography system. The column was equilibrated and then developed at a flow rate of 4 ml/min with a solution containing 10 mM phosphate buffer (pH 7.5) and 0.1 M KCl. Fractions were collected every 0.5 min, and then 80% glycerol (final concentration, 9%) was added to each fraction. The methylation activity for DMST, ST, DHDMST, and DHST in the fractions was measured. Dextran blue (2,000 kilodaltons), β-amylase (200 kilodaltons), alcohol dehydrogenase (150 kilodaltons), and bovine serum albumin (66 kilodaltons) were also applied to the column to calibrate the molecular mass. The protein concentration was determined by the method of Bradford (6).

RESULTS

Aflatoxin production from various ST derivatives. *A. parasiticus* NIAH-26 was incubated in YES medium with DMST, DHDMST, dimethoxy-ST, 5-methoxy-ST, or sterigmatin (Fig. 2A). When this mutant was cultured with DMST, two fluorescent spots (one blue and one green) were formed whose R_f values corresponded to those of authentic AFB_1 and AFG_1. When DHDMST, which is a dihydro derivative of DMST, was added to the medium, AFB_2 and AFG_2 were produced. Similar aflatoxin production from DMST and DHDMST was also observed in the feeding experiments when other mutants were used instead of NIAH-26 (15) (data not shown). Moreover, a small amount of AFB_1 and AFG_1 was formed by feeding 5-methoxy-ST to this mutant. However, no aflatoxins were produced in the presence of either dimethoxy-ST or sterigmatin.

The enzymatic reactions in the cell-free system were examined (Fig. 2B). When the cell extract of this mutant was incubated with DMST in the presence of nonradiolabeled SAM, a yellow fluorescent spot appeared which was determined to be OMST by comparison with the standard sample. Also, incubation with DHDMST in the presence of SAM led to the production of DHOMST. When NADPH was also present, AFB_1 and AFB_2 were produced from DMST and DHDMST, respectively (data not shown). On the other

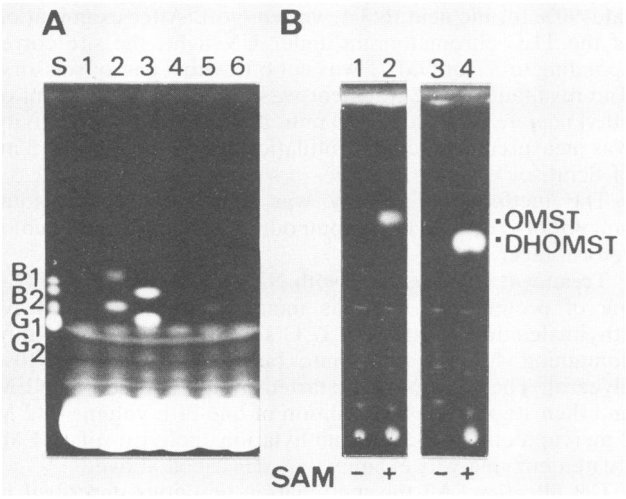

FIG. 2. Production of aflatoxins from DMST or DHDMST in feeding and cell-free experiments. (A) The mutant *A. parasiticus* NIAH-26 was cultured in YES medium (lane 1) or YES medium containing 100 μM DMST (lane 2), DHDMST (lane 3), dimethoxy-ST (lane 4), 5-methoxy-ST (lane 5), or sterigmatin (lane 6). After 4 days of culture, 10 μl of each culture medium was analyzed by TLC. No significant difference in the mycelial wet weight was found among the experiments. (B) Cell extract (2.0 mg of protein per ml) was incubated with either 90 μM DMST (lanes 1 and 2) or DHDMST (lanes 3 and 4) at 37°C for 30 min. SAM (0.3 mM) was also added when specified. Reaction products were analyzed by TLC with chloroform–ethylacetate–90% formic acid (6:3:1, vol/vol/vol). Fluorescence photographs are shown.

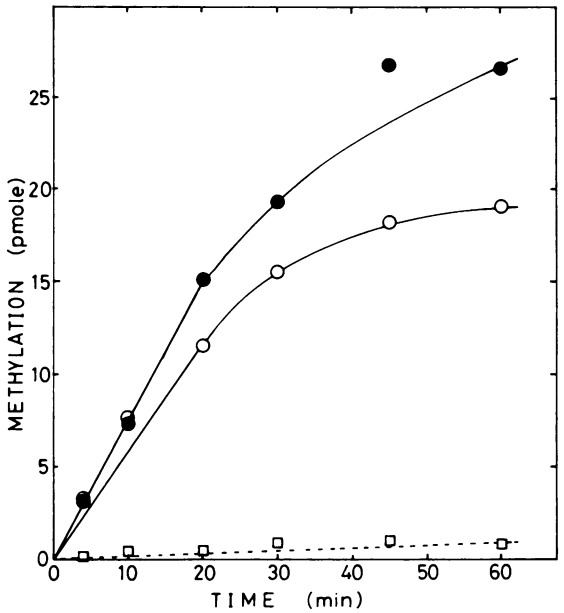

FIG. 3. Methylation of DMST and ST. Cell extract (0.2 mg of protein per ml) was incubated with 10 μM [^{3}H]SAM in the presence of 50 μM each DMST (●), ST (○), and OMST (□). The radioactivity of tritium transferred from the reaction mixture to the chloroform layer was measured.

hand, no accumulation of ST or DHST was detected in this assay. When the extract was incubated with 5-methoxy-ST, dimethoxy-ST, or sterigmatin, no new products were detected even in the presence of either SAM or NADPH or both (data not shown).

Methylation of DMST, ST, DHDMST, and DHST. To analyze the methylation reaction in more detail, we examined the incorporation of the methyl group of [^{3}H]SAM into DMST, DHDMST, ST, and DHST. When either DMST or ST was incubated with [^{3}H]SAM, the radioactivity of tritium transferred to the chloroform layer increased significantly with time (Fig. 3). However, when OMST was incubated with [^{3}H]SAM, tritium was scarcely transferred to the chloroform layer.

The methylation of various STs was also observed (Table 1). When either DHDMST or DHST was incubated with [^{3}H]SAM, tritium was transferred to the chloroform layer. In contrast, no methylation activity was observed when DHOMST, 5-methoxy-ST, dimethoxy-ST, or sterigmatin was used as the substrate. Also, no methylation reactions were found in the cell extract obtained from the same mutant cultured in YEP medium (data not shown). The products of these methylation reactions were then subjected to TLC analyses. Figure 4 gives the time course of the methylation of DMST, showing that tritium from [^{3}H]SAM was incorporated into the ST fraction and that the extent of the incorporation reached a maximum value after a short interval and then remained constant, whereas the accumulation of tritium in the OMST fraction continued to increase. The recovery of radioactivity spotted onto the TLC plate was nearly 100%. These results indicate that DMST is first converted to ST and then ST is converted to OMST by further methylation. When DHDMST was used instead of DMST, the *methyl*-^{3}H group of [^{3}H]SAM was also at first transferred to the DHST fraction and then to the DHOMST fraction (data not shown).

Effect of NEM treatment of cell extract on methyltransferase activities. The cell extract was modified by NEM, a reagent for modification of protein SH groups, and the methyltransferase activities of the resultant extract were examined (Fig. 5). The methylation activity for DMST was significantly decreased by the NEM treatment to approximately 4% of the original value. In contrast, the methylation activity for ST remained almost constant (approximately 82% of the original value), even when a NEM-treated extract was used. Also, the NEM treatment of the cell extract caused a significant inhibition of the methylation activity for DHDMST (to 4% of the original value) but not of that for DHST (to 78% of the original value).

Gel filtration of postmicrosomal fraction. The methylation activities for DMST, ST, DHDMST, and DHST were measured in each fraction obtained by gel filtration (Fig. 6). The methylation activities for DMST and DHDMST peaked at the same elution time (40.5 min), corresponding to approxi-

TABLE 1. Substrate specificity of methyltransferase

Substrate[a]	Methylation[b] (pmol/20 min)
DMST	14.7
ST	11.8
OMST	0.5
DHDMST	14.5
DHST	11.7
DHOMST	0
Dimethoxy-ST	0
5-Methoxy-ST	0
Sterigmatin	0

[a] Cell extract was incubated with 50 μM substrate at 37°C for 20 min.
[b] Value represents the means of duplicate experiments.

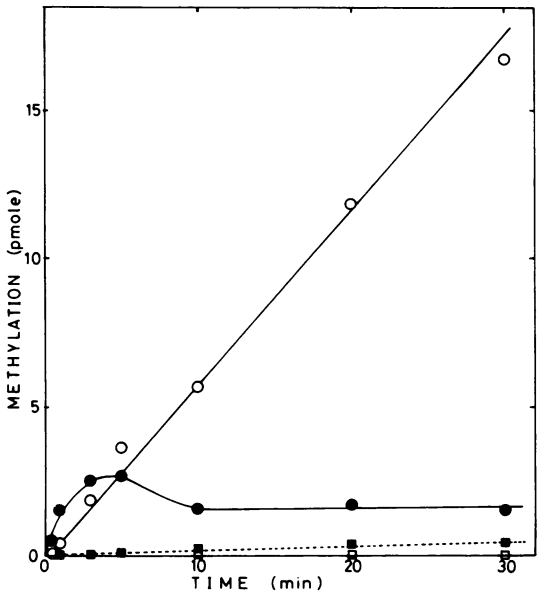

FIG. 4. Incorporation of the *methyl*-^{3}H group of [^{3}H]SAM to ST and OMST. Cell extract (0.2 mg of protein per ml) was incubated with 10 μM [^{3}H]SAM in the presence (○, ●) or absence (□, ■) of 50 μM DMST. At the times indicated, the reaction was stopped by the addition of chloroform, and the radioactivity of tritium in the chloroform layer was analyzed by TLC. The radioactivity of tritium incorporated into ST (●, ■) or OMST (○, □) was measured as described in Materials and Methods.

mately 210-kilodalton molecules. The accumulated products from the methylation of DMST and DHDMST were OMST and DHOMST, respectively (data not shown). The recovery of the methylation activities was about 30% for both substrates. Methylation activities for ST and DHST also peaked at the same time (41.5 min), corresponding to about 180-kilodalton molecules, and 60% of both activities were recovered after elution. The products of ST and DHST were OMST and DHOMST, respectively (data not shown).

DISCUSSION

Our previous study demonstrated that AFB_1-AFG_1 and AFB_2-AFG_2 are independently produced from ST and DHST, respectively, by a common enzyme system (15). The present study also demonstrates that DMST and DHDMST are immediate precursors in the formation of ST and DHST, respectively. The metabolic scheme proposed for the late stages of aflatoxin biosynthesis is shown in Fig. 7. Two kinds of *O*-methyltransferases are designated as MT-I and MT-II, respectively, in Fig. 7.

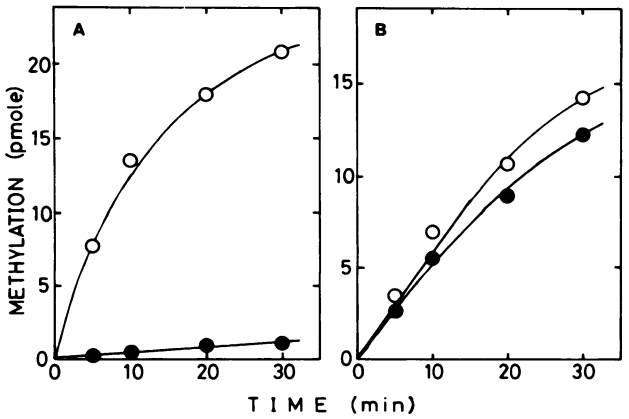

FIG. 5. Effect of NEM treatment of the cell extract on its methylation activity for DMST or ST. The cell extract was incubated with (●) or without (○) NEM. The methylation activity of the resultant extract for DMST (A) or ST (B) was then analyzed.

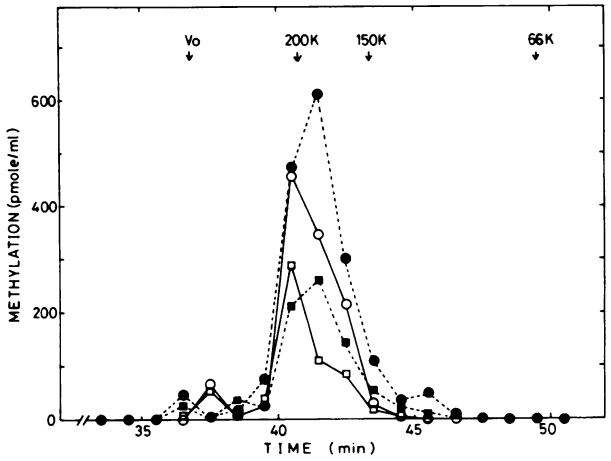

FIG. 6. Gel filtration chromatography for methylation activity. Each fraction (28 μl) was incubated with 50 μM each DMST (○), ST (●), DHDMST (□), or DHST (■) in the reaction mixture (final volume, 50 μl) at 37°C for 60 min. After termination of the reaction by the addition of chloroform, the radioactivity in the chloroform layer was measured. The molecular weight standards are shown in thousands (k) (see text).

DMST and DHDMST contain two free hydroxyl groups, C-7-OH and C-6-OH, whereas ST and DHST contain one hydroxyl group, C-7-OH. The methoxy group of AFB_1, which corresponds to the methoxy group at the C-6 position of ST, has been reported to be derived from methionine (5). In the present study, tracer experiments with [^{3}H]SAM followed by TLC analysis showed that the C-6-OH group of DHST and DHDMST is first methylated by *O*-methyltransferase I and then the C-7-OH group is methylated by *O*-methyltransferase II. When the *O*-methyltransferase I activity was inhibited by NEM, *O*-methyltransferase II, whose activity remained intact, could not methylate DMST (Fig. 5A). Therefore, the C-6-OH methylation catalyzed by *O*-methyltransferase I is a prerequisite for the subsequent C-7-OH methylation catalyzed by *O*-methyltransferase II.

On the other hand, the effect of NEM modification on the *O*-methyltransferase-catalyzed conversion of DMST to ST was indistinguishable from that on the *O*-methyltransferase-catalyzed conversion of DHDMST to DHST. In contrast, the *O*-methyltransferase II activity which catalyzes the

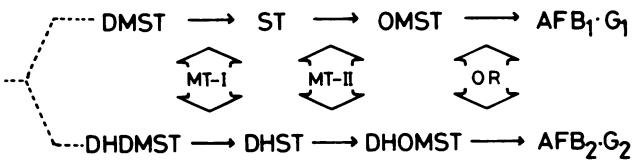

FIG. 7. Metabolic scheme proposed for the late stages of aflatoxin biosynthesis. ——, Confirmed reactions (see text and reference 15); – – – –, hypothetical reactions. Abbreviations: MT-I, *O*-methyltransferase I; MT-II, *O*-methyltransferase II; OR, oxidoreductase.

reaction from ST to OMST, as well as that which catalyzes the reaction from DHST to DHOMST, was insensitive to the SH modification by NEM. In the gel filtration experiment (Fig. 6), the peak of the methylation activity with DMST as the substrate was inseparable from that with DHDMST as the substrate. Also, the peak of the methylation activity with ST was inseparable from that with DHST. Therefore, it is considered that the two distinct *O*-methyltransferases I and II are involved in both the DMST-to-ST-to-OMST and the DHDMST-to-DHST-to-DHOMST conversions. Hence, the ratio of AFB_1 and AFG_1 to AFB_2 and AFG_2 may be determined by the relative concentration of DMST and DHDMST, which may also be regulated in any of the steps preceding the formation of these compounds.

O-Methyltransferases I and II were different in their protein molecules, and their molecular masses were calculated to be 210 and 180 kilodaltons, respectively, by gel filtration. However, since no reduced agent such as mercaptoethanol was added to the elution medium in our experiment, the large molecules obtained may have originated from complexed forms of some subunits. Furthermore, the activity of *O*-methyltransferase I toward DMST and DHDMST is sensitive to NEM modification, whereas that of *O*-methyltransferase II toward ST and DHST is not, which implies that there are additional structural differences between the enzymes.

The results listed in Table 1 suggest that both *O*-methyltransferases I and II show a strict substrate specificity to the STs, although they cannot discriminate between the dihydrobisfuran and tetrahydrobisfuran structures. 5-Methoxy-ST and dimethoxy-ST contain free C-7-OH, but they could not serve as substrates for the methylation reaction. Sterigmatin contains two free OH groups, as in the case of DMST and DHDMST, but could not serve as substrate either, suggesting that *O*-methyltransferases I and II may be exclusively related to aflatoxin biosynthesis.

5-Methoxy-ST was first isolated by Holker and Kagel from the *A. versicolor* mutant, which does not produce significant quantities of ST, whereas the parent strain of this mutant produces relatively large quantities of ST and only traces of the 5-methoxy derivative (13). They suggested that 5-methoxy-ST may be the precursor of ST or that 5-hydroxysterigmatocystin (5-hydroxy-ST) may be a common precursor of 5-methoxy-ST and ST. In our feeding experiments with 5-methoxy-ST, a small amount of AFB_1 and AFG_1 was produced by the mutant NIAH-26, but not by the other 26 kinds of mutants (15) (data not shown). Also, neither aflatoxins nor other substances were detected in the cell-free experiments when the cell extract of the mutant NIAH-26 was incubated with 5-methoxy-ST in the presence of SAM and/or NADPH. Elsworthy et al. reported that a synthetic 5-hydroxydihydrosterigmatocystin (5-hydroxydihydro-ST), a dihydro derivative of 5-hydroxy-ST, may be a precursor of AFB_2 and AFG_2 in *A. parasiticus* (9). The biosynthetic relationship between 5-methoxy-ST and aflatoxin biosynthesis remains unclear.

Schroeder and Kelton (14) examined the production of ST by common storage fungi and showed that ST is not always found in aflatoxin-producing molds. Our results (Fig. 4) show that the fact that ST could not be isolated from aflatoxin-producing molds may suggest that ST produced from DMST by *O*-methyltransferase I is immediately converted to OMST by *O*-methyltransferase II.

Our results also demonstrate that the activities of *O*-methyltransferases I and II are not detected in aflatoxin-noninducible medium. The previous report also showed that the oxidoreductase was not produced in that medium (15). Abdollahi and Buchanan reported that the initiation of aflatoxin synthesis could be blocked by treatment of the mold with cycloheximide or actinomycin D (1). The expression of the *O*-methyltransferases and the oxidoreductase system shown in Fig. 7 may be commonly regulated at the transcriptional level by an unidentified factor(s) depending on the kinds of carbon sources.

After this manuscript had been submitted, Bhatnagar et al. (4) reported that a methyltransferase which catalyzes the reaction from ST to OMST was purified to homogeneity, that it had a native molecular mass of 160 kilodaltons, and that it appeared to have subunits of 110 and 58 kilodaltons. This enzyme may correspond to *O*-methyltransferase II described in this paper.

ACKNOWLEDGMENTS

We thank N. Terakado, National Institute of Animal Health, Ministry of Agriculture, Forestry and Fisheries, for critically reviewing this manuscript. We also thank S. Hara, National Institute of Animal Industry, for his valuable discussions and S. Shimizu and T. Watanabe, National Institute of Animal Health, for their help in parts of the experiments.

This work was supported in part by a Grant-in-Aid (Bio Media Program) from the Ministry of Agriculture, Forestry and Fisheries (BMP 89-III-2-1).

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