Aflatoxin Biosynthesis and Genes of *Aspergillus parasiticus*

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Abstract

Aflatoxins are produced from acetyl CoA through more than 18 enzyme steps. Although most of all enzymatic reactions have already been reproduced by *in vivo* and *in vitro* experiments, a few enzyme activities such as one catalyzing reaction from *O*-methylsterigmatocystin to aflatoxin G₁ remained to be clarified.

We recently detected biosynthetic activities for aflatoxins G₁ and G₂ in cell-free extracts of *A. parasiticus* NIAH-26. In the presence of NADPH, aflatoxins G₁ and G₂ were produced from *O*-methylsterigmatocystin and dihydro-*O*-methylsterigmatocystin, respectively. Also, we for the first time determined that G- and B-aflatoxins are independently produced from the same substrate. At least three reactions, catalyzed respectively by the *ordA* gene product, an unstable microsome enzyme, and an 220-kDa cytosol protein, are involved in the enzymatic formation of G-aflatoxins from either *O*-methylsterigmatocystin or dihydro-*O*-methylsterigmatocystin.

On the other hand, many of the genes involved in aflatoxin biosynthesis have been isolated from *A. parasiticus* and *A. flavus*, and most of them have been found to be clustered. We recently purified *O*-methyltransferase catalyzing the conversion of demethylsterigmatocystin to sterigmatocystin in aflatoxin biosynthesis. Based on the N-terminal amino acid sequence of the enzyme, genomic and cDNA genes (*dmtA*) of the enzyme were then cloned and sequenced from *A. parasiticus* NIAH-26 through the use of polymerase chain reaction (PCR) strategies. The *dmtA* gene was found to be placed between the *omtA* and *ord-2* genes in the aflatoxin biosynthesis cluster region with the same orientation. Clarification of the aflatoxin biosynthetic mechanism using the biochemical and molecular biological approaches will provide promising clues to eliminate aflatoxin contamination from crops.

Key words: *Aspergillus parasiticus*, aflatoxin, biosynthesis

Introduction

Aflatoxins B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁), and G₂ (AFG₂) are toxic, carcinogenic secondary metabolites produced by some strains of *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, and *A. tamarii*. Contamination of feed and food with aflatoxins can have serious effect on health of animals and human. Many researchers have studied aflatoxin biosynthetic mechanism for the purpose of development of methods for preventing aflatoxin contami-

nation.

All aflatoxins are produced from acetyl CoA through complicated pathways consisting of more than 18 enzyme steps¹⁾. For the determination of the pathways, two kinds of methods, *in vivo* feeding experiment and *in vitro* enzyme assay, have been mainly used. Although most of all reaction steps had already been determined by the both *in vivo* and *in vitro* experiments, a few enzyme activities such as ones catalyzing the conversion of acetyl CoA to hexanoyl CoA, hexanoyl CoA to norsolorinic acid, averufin to versiconal hemiacetal acetate, versicolorin A to demethylsterigmatocystin, and *O*-methylsterigmatocystin (OMST) to AFG₁ remained to be clarified.

In this study, we investigated the enzyme activity for the formation of G-group aflatoxins in detail²⁾. The biosynthetic pathway(s) associated with the formation of G-group aflatoxins has long been controversial. Some researchers have reported that in feeding experiments radioactive AFB₁ was converted to G-group aflatoxins, while others have not observed this³⁾. However, it is generally assumed that G-group aflatoxins are produced from B-aflatoxins by insertion of oxygen into a C-C bond through a Baeyer-Villiger reaction. We previously reported enzymatic conversion of OMST to AFB₁ or dihydro-*O*-methylsterigmatocystin (DHOMST) to AFB₂ by using cell-free extract of *Aspergillus parasiticus* NIAH-26 and thin layer chromatography analysis⁴⁾. In the course of the similar experiments using OMST as a substrate, faint bands corresponding to AFG₁ together with clear bands of AFB₁ were sometimes observed. Therefore, in order to confirm the production of G-group aflatoxins in more detail, we used HPLC analyses for the cell-free assays. Finally, we determined the enzyme activity for G-group aflatoxins.

On the other hand, many genes of the enzymes and the transcription factor involved in aflatoxin biosynthesis have been cloned and characterized, following determination of the biosynthetic pathways⁵⁾. These genes have been shown to form a gene cluster located in an approximately 75-kb region in the *A. flavus* and *A. parasiticus* genomes, although some differences between different species exist. Furthermore, many fungi such as *A. nidulans* and *Bipolaris* produce sterigmatocystin (ST), a precursor of aflatoxins, and it is suggested that almost the same pathways as that for aflatoxin biosynthesis may also be involved in sterigmatocystin biosynthesis; in fact, similar genes as those in the aflatoxin biosynthesis have been isolated from *A. nidulans*, and it has been found that these genes are also located in a 60-kb cluster of the *A. nidulans* genome⁶⁾. Determination of enzyme activity makes it possible to isolate the gene coding the enzyme. Thus, we determined the gene (named as *dmtA*) coding *O*-methyltransferase I (MT-I) catalyzing the conversion of demethylsterigmatocystin to sterigmatocystin and of dihydrodemethylsterigmatocystin to dihydrosterigmatocystin⁷⁾.

Materials and Methods

Microorganisms and cultures *A. parasiticus* NIAH-26, a UV-irradiated mutant of *A. parasiticus* SYS-4 (NRRL 2999) was used⁸⁾. Fungal strains were cultured at static state at 28°C for 4 days either in a YES liquid medium, an aflatoxin-inducing medium, or in a YEP liquid medium (2% yeast extract, 20% peptone), an aflatoxin-non-inducing medium.

Enzyme assays for the aflatoxin formation Cell-free extract, microsome, and cytosol fractions were prepared from the mycelia of *A. parasiticus* NIAH-26 grown in YES medium

at 28°C for 4 days by grinding the mycelia and then successive centrifugations⁹). We devised assay conditions for optimizing the enzyme activity. The cell-free extracts (1.3 mg of protein per ml) was incubated in a reaction mixture consisting of 90 mM potassium phosphate [pH 7.5], 10% [vol/vol] glycerol supplemented with 80 μ M of each substrate, 4 mM NADPH, BSA (0.9 mg/ml), and phosphatidyl choline liposome (0.5 mg/ml). The final volume of the reaction mixture was 50 μ l. Reactions were routinely carried out at 24°C for 40 min and then terminated by adding water-saturated chloroform and mixing with a Vortex mixer. An aliquot of the lower chloroform layer was directly injected into a Shimadzu LC-6A HPLC equipped with a silica gel HPLC column. The solvent system consisted of toluene/ethyl acetate/formic acid/methanol (198: 15: 4: 3, v/v/v/v). The fluorescence intensity of each aflatoxin was monitored with a fluorescence monitor (ex. was 365 nm; em. was 425 nm) with a flow rate of 1 ml/min at room temperature.

Determination of the amino acid sequence of the MT-I MT-I was purified as described previously¹⁰, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoreses using 13% polyacrylamide gel. The protein was blotted onto a polyvinylidene difluoride membrane (immobilon P), and the part corresponding to MT-I in the membrane was cut and then applied onto an automated Edman degradation gas phase sequencer.

cDNA synthesis and 5'- and 3'-RACE PCR To obtain the full-length cDNA sequence, mRNA (1 μ g) was used to generate double-stranded cDNA using the Marathon cDNA Amplification kit (CLONTECH Laboratory, Inc., Palo Alto, CA), and the double-stranded cDNA was ligated with the Marathon cDNA adaptor. 3'-RACE and 5'-RACE were performed using the double-stranded cDNA as a template with a *dmtA* gene-specific primers and the adaptor primer AP1. The PCR products of both 5'-RACE and 3'-RACE were gel-purified, ligated into vector TA, and sequenced.

Cloning of genomic DNA and TAIL-PCR DNA was purified from *A. parasiticus* NIAH-26. To determine the internal sequence of the genomic *dmtA* gene, PCR reactions were carried out using the genomic DNA of *A. parasiticus* NIAH-26 as a template with various primers having the cDNA sequence, and the resultant PCR products were sequenced. To determine the sequences of the 5'- as well as the 3'-flanking regions, thermal asymmetric interlaced (TAIL) PCR¹⁰ was used.

Northern analysis The spore suspension was inoculated into 100 ml of either SY liquid medium (6% sucrose, 2% yeast extract) as an aflatoxin-inducing medium or PY liquid medium (4% peptone and 2% yeast extract) as an aflatoxin-non-inducing medium, and incubated at either 28°C, permissive temperature of aflatoxin production, or at 37°C, non-permissive temperature, for 3 days on a rotary shaker, and then the mycelia were harvested. The membranes the total RNA was blotted onto were then probed with the DIG-labeled DNA fragment, and the hybridized DNA band was immunodetected by using the Detection DIG Luminescent Detection Kit (Boehringer Mannheim).

DNA sequence analysis. All PCR products were ligated into TA-cloning vector, pCR 2.1, and then transformed into bacteria, INVaF (Invitrogen BV, The Netherlands), and sequenced. The genomic and cDNA nucleotide sequence data of *dmtA* are available from DDBJ/

EMBL/GenBank nucleotide sequence databases with accession numbers AB022905 and AB022906.

Results

Detection of enzyme activity for G-group aflatoxin formation When the cell-free extract of *A. parasiticus* NIAH-26 was incubated with OMST in the presence of NADPH at 24°C for 40 min, AFG₁ as well as AFB₁ were formed. When DHOMST, instead of OMST, was added to the reaction mixture, AFG₂ and AFB₂ were produced. The amount of AFG₁ produced from OMST continued to increase for at least 40 min. We found that co-presence of lipid (0.5 mg/ml) and BSA (0.9 mg/ml) in the reaction mixture maximized the enzyme activity. The formation of AFG₁ and AFB₁ was reduced by 3 mM of methyrapone, SKF-525A, or imidazole, indicating that the formation of both AFB₁ and AFG₁ were dependent upon cytochrome P-450 oxygenase activity.

The enzyme activity for G-AF formation using various metabolites as substrates were measured. AFG₁ was produced from OMST, and AFG₂ was from DHOMST. However, neither AFG₁ nor AFG₂ was produced from either AFB₁ or AFB₂, nor did the reverse reaction from G-group aflatoxins to B-group aflatoxins occurred. These results indicate that B-group aflatoxins are not the precursors of G-group aflatoxins, and that G- and B-group aflatoxins are independently produced from the same substrate (OMST for AFG₁ and AFB₁, and DHOMST for AFG₂ and AFB₂) through different pathways from a common branching intermediate.

To determine the relationship between AFG₁ and AFG₂ formation, we examined the enzyme activities by using reaction mixtures that contained 5 μ M DHOMST and different concentrations of OMST. Although AFG₂ was produced from DHOMST, the amount formed decreased with increasing OMST concentration and AFG₁ newly formed. Addition of high concentrations of OMST (more than 10 μ M) led to exclusive formation of AFG₁. These results indicate that the same enzyme(s) may catalyze the formation of AFG₁ and AFG₂.

Requirement of both the microsome fraction and 220-kDa protein for G-aflatoxin production We measured the G-aflatoxin formation activity by using either microsome or cytosol fractions or a combination of both. No significant amounts of AFG₁ were formed in the OMST+cytosol reaction, and the OMST+microsome reaction produced only trace of AFG₁. When both the cytosol and microsome fractions were present, enzyme activity was markedly enhanced and dependent upon microsome concentration. We examined the activation factor in the cytosol fraction by fractionating it through an Ultrogel Aca 34 gel filtration column and then incubating each fraction with OMST in a reaction mixture containing the microsome fraction. AFG₁ formation activity was enhanced threefold by the addition of the fraction that corresponded to a molecular weight of about 220 kDa. These results indicate that at least two kinds of enzymes, one a microsome enzyme and the other a 220-kDa soluble protein, are necessary for the formation of AFG₁ from OMST.

When the cell-free extract was incubated with or without liposome and BSA at 24°C for various times, AFG₁ production decreased to less than 50% of the original activity level after 15 min and to less than 20% after 30 min, whereas the AFB₁ formation activity was more stable than was that for AF. It was found that the microsome enzyme is the cause of

instability observed for G-group aflatoxin biosynthesis.

Cloning and nucleotide sequencing of MT-I cDNA and its deduced amino acid sequence

We purified MT-I from *A. parasiticus* NIAH-26 to homogeneity. Its molecular weight was 43 kDa (SDS-PAGE) and the *N*-terminal sequence was: T-G-L-D-M-E-I-I-F-A-K-I-K-E-E-Y-A-R-T-D-D-V-G-K-R-Q-I-Q-G-H-I-R-E-L-Q-V-G-F-Y-S(P)-D-L(W)-D-V-V-M-R-L-A(S)-S-G-. In order to determine the specific nucleotide sequence of *dmtA*, we used degenerate oligonucleotides corresponding to the *N*-terminal sequence of the MT-I protein. A 144-bp PCR product was obtained.

We then used 5'-RACE and 3'-RACE techniques to determine the cDNA sequence of *dmtA* gene. Conventional and TAIL-PCR were further used to determine the genomic DNA sequence⁷⁾. The overall cDNA sequence was determined to be 1373 bp, and first M may later be processed post-translationally. The open reading frame of the cDNA codes for a 386-amino-acid-residue protein, and the theoretical molecular mass without the *N*-terminal methionine was calculated to be 43,023 Da, which corresponded well with the mass determined by SDS-PAGE (43 kDa)¹⁰⁾. DmtA (=MT-I) contains a consensus motif I of *S*-adenosylmethionine-dependent methyltransferase in the central region.

By comparison of the cDNA sequence with the genomic DNA sequence, the coding sequence (nucleotide 1/1333) of *dmtA* was found to be separated with three introns. In addition, the palindrome sequence TCG(N5)CGA, which has been suggested to be a possible binding region of AflR in *A. nidulans*¹²⁾, was found. Three TATAA sequences close to the transcription initiation site were also found.

Using TAIL-PCR, it was found that the sequence of the region of -703/-499 overlapped with the 3'-flanking region of the *omtA* gene. We also found that the 3'-flanking region (1415/1683) of *dmtA* overlapped with the 5'-cDNA sequence of *ord-2*. The orientations of *dmtA* and *ord-2* were also the same.

Expression of the *dmtA* gene Transcription of *dmtA* gene was determined by Northern blotting. When *A. parasiticus* NIAH-26 was cultured at 28°C for 3 days in an aflatoxin-inducing SY medium, a 1.4-kb transcript of *dmtA* was seen. In contrast, when an aflatoxin-non-inducing PY medium was used instead of SY, *dmtA* was not expressed. Also, when the fungi were cultured at a higher temperature (37°C), an aflatoxin-non-permissive temperature¹³⁾, the expression of *dmtA* was not detected, irrespective of the kind of medium.

Discussion

We detected enzyme activity for G-aflatoxin formation by using a cell-free extract or a combination of cytosol and microsome fractions of *A. parasiticus* NIAH-26. The relative week activity of G-aflatoxin biosynthesis may indicate that the enzymes for G-aflatoxin formation are relatively sensitive to the preparation of the cell-free fractions, whereas the enzymes for B-aflatoxin formation are much less sensitive to this process. Furthermore, G-aflatoxin biosynthesis requires both cytosol and microsome fractions and, if only one of these fractions was used for examining G-aflatoxin biosynthesis, then no G-aflatoxin would have been detected. These findings may at least partly explain why the enzyme activity for G-aflatoxin biosynthesis had not been previously detected.

In this study, we for the first time determined independent formation of G- and B-group

Fig. 1 Metabolic pathway for formation of AFG₁ from OMST and formation of AFG₂ from DHOMST.

At least three enzyme reactions may be commonly included in both formations. Reaction 1 may be catalyzed by an *orda* gene product, which is microsomal cytochrome P-450 oxygenase. A certain transient intermediate formed during this pathway may be subsequently converted to another substance in reaction 2, and finally converted to the final product, AFG₁ or AFG₂ in reaction 3. Reactions 2 and 3 may be catalyzed by an unstable microsomal enzyme and a 220-kDa cytosolic protein. However, the order of these three enzymes was not determined in this study.

aflatoxins. AFG₁ and AFG₂ were produced from OMST and DHOMST, respectively, whereas neither AFG₁ nor AFG₂ was produced from AFB₁ or AFB₂ in the cell-free systems. Recently, *ord1* gene and *orda* gene were isolated from *A. flavus*¹⁴⁾ and from *A. parasiticus*¹⁵⁾, respectively, and were confirmed to be required for the conversion of OMST to AFB₁. Yu *et al.*¹⁵⁾ reported that complementation of *A. parasiticus* SRRRC 2043, an OMST-accumulating strain, with the *orda* gene restores the ability to produce AFB₁, AFB₂, AFG₁, and AFG₂, indicating that *orda* gene product may be needed for the formation of G-group aflatoxins.

However, our results show differences between the biosynthetic activities for G-AF and B-AF, because the microsomal enzyme fraction involved in G-formation was less stable than that for B-formation. Therefore, at least three enzymes appeared to be required for conversion of OMST to AFG₁ and that of DHOMST to AFG₂ (Fig. 1): a microsomal *orda* gene product, an unstable microsomal protein, and a 220-kDa cytosolic protein. Although B-aflatoxins may be the final products of the reactions catalyzed by the *orda* product, more than two reactions may be included during the pathway either from OMST to AFB₁ or from DHOMST to AFB₂.

This study gives a final conclusion to long controversy about G-aflatoxin biosynthesis. It is generally accepted that strains of *A. parasiticus* produce both G- and B-aflatoxins, while

A. flavus strains produce only the B-aflatoxins. Also, *A. nidulans* does not make aflatoxins, because sterigmatocystin and dihydrosterigmatocystin are their final products. This study is showing a clue to elucidate the differences in aflatoxin productivity among the fungi.

On the other hand in the current study, we determined the cDNA and genomic DNA sequences encoding *O*-methyltransferase I from *A. parasiticus*. This gene is present as a single-copy in the genome, and is placed between the *omtA* and *ord-2* genes within the aflatoxin biosynthesis-gene cluster.

Kelkar *et al*¹⁶⁾ have found by gene disruption strategy that an open reading frame from 42597 to 41970 (ENU 34740) of the *A. nidulans stcP* gene encodes MT-I. Since the *dmtA* gene of *A. parasiticus* is supposed to be a homolog of the *stcP* gene, the deduced amino acid sequences of both genes were compared. The *dmtA* gene product showed a 74.4% identity and 87% similarity to the deduced gene product encoded by a nucleotide sequence of the 1289-bp region (43273-41985), which covers the region containing 5'-flanking region of the *stcP* gene and the gene itself. This *stcP* whole region may code a protein composed of 424 amino acids, and its theoretical molecular mass is 47,840 Da.

Gene walking using TAIL-PCR technology revealed that the *dmtA* gene is placed between the *omtA* gene and the *ord-2* genes in the same direction. The region between *omtA* and *dmtA* contains the TATA sites and the AflR recognition site¹²⁾, indicating that the expression of *dmtA* is also regulated by the aflR gene product. This was also confirmed by the results showing that the expression of *dmtA* has the same dependency on the culture temperature and the culture medium.

At least a part of the aflatoxin biosynthetic pathways has commonly been detected in many species. Although *A. flavus* and *A. parasiticus* can be expected to have whole aflatoxin gene cluster leading to the formation of aflatoxins, other fungi such as *A. nidulans* contain genes until sterigmatocystin formation and so on. The each coding region of each gene are similar among the different fungi, whereas the distribution of each genes and the non-coding regions are remarkably different. Evolutional relationship among the genes seems to be interesting. Based on these knowledge, it will be possible that the useful methods for prevention of the aflatoxin contamination will be obtained in the near future.

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References

- 1) Yabe, K. (1998) Biosynthetic pathways of aflatoxins. Nippon Nogeikagaku Kaishi. 72: 63-67 (in Japanese).
- 2) Yabe, K., Nakamura, M., Hamasaki, T. (1999) Enzymatic formation of G-group aflatoxins and biosynthetic relationship between G- and B-group aflatoxins. Appl. Environ. Microbiol. 65: 3867-3872.
- 3) Henderberg, A., Bennett, J. W., L. S. Lee. (1988) Biosynthetic origin of aflatoxin G₁: confirmation

- of sterigmatocystin and lack of confirmation of aflatoxin B₁ as precursors. J. Gen. Microbiol. 134: 661-667
- 4) Yabe, K., Ando, Y., Hamasaki, T. 1988. Biosynthetic relationship among aflatoxins B₁, B₂, G₁, and G₂. Appl. Environ. Microbiol. 54: 2101-2106.
 - 5) Woloshuk, C. P., Prieto, R. 1998. Genetic organization and function of the aflatoxin B₁ biosynthetic genes. FEMS Microbiology Letters 160: 169-176.
 - 6) Brown, D. W., Yu, J.-H., Kelkar, H. S., Fernandes, M., Nesbitt, T. C., Keller, N. P., Adams, T. H., Leonard, T. J. (1996) Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA 93: 1418-1422.
 - 7) Motomura, M., Chihaya, N., Shinozawa, T., Hamasaki, T., Yabe, K. (1999) Cloning and characterization of the *O*-methyltransferase I gene (*dmtA*) from *Aspergillus parasiticus* associated with the conversions of demethylsterigmatocystin to sterigmatocystin and dihydrodemethylsterigmatocystin to dihydrosterigmatocystin in aflatoxin biosynthesis. Appl. Environ. Microbiol. 65: 4987-4994.
 - 8) Yabe, K., Nakamura, H., Ando, Y., Terakado, N., Nakajima, H., Hamasaki, T. (1988) Isolation and characterization of *Aspergillus parasiticus* mutants with impaired aflatoxin production by a novel tip culture method. Appl. Environ. Microbiol. 54: 2096-2100.
 - 9) Yabe, K., Ando, Y., Hamasaki, T. (1991) A metabolic grid among versiconol hemiacetal acetate, versiconol acetate, versiconol and versiconol during aflatoxin biosynthesis. J. Gen. Microbiol. 137: 2469-2475.
 - 10) Yabe, K., Matsushima, K.-I., Koyama, T., Hamasaki, T. (1998) Purification and characterization of *O*-methyltransferase I involved in the conversion of demethylsterigmatocystin to sterigmatocystin and of dihydrodemethylsterigmatocystin to dihydrosterigmatocystin during aflatoxin biosynthesis. Appl. Environ. Microbiol. 64: 166-171.
 - 11) Liu, Y.-G., Whittier, R. F. (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert and fragments from P1 and YAC clones for chromosome walking. Genomics 25: 674-681
 - 12) Fernandes, M., Keller, N. P., Adams, T. H. (1998) Sequence-specific binding by *Aspergillus nidulans* AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. Mol. Microbiol. 28: 1355-1365.
 - 13) Feng, G. H., Leonard, T. J. (1998) Culture conditions control expression of the genes for aflatoxins and sterigmatocystin biosynthesis in *Aspergillus parasiticus* and *A. nidulans*. Appl. Environ. Microbiol. 64: 2275-2277.
 - 14) Prieto, R., Woloshuk (1997) *ord1*, an oxidoreductase gene responsible for the conversion of *O*-methylsterigmatocystin to aflatoxin in *Aspergillus flavus*. Appl. Environ. Microbiol. 63: 1661-1666.
 - 15) Yu, J., Chang, P.-K., Payne, G. A., Cary, J. W., Bhatnagar, D., Cleveland T. E. (1995) Comparison of the *omtA* genes encoding *O*-methyltransferases involved in aflatoxin biosynthesis from *Aspergillus parasiticus* and *A. flavus*. Gene 163: 121-125.
 - 16) Kelkar, H. S., Keller, N. P., Adams, T. H. (1996) *Aspergillus nidulans stcP* encodes an *O*-methyltransferase that is required for sterigmatocystin biosynthesis. App. Environ. Microbiol. 62: 4296-4298.