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Participation in aflatoxin biosynthesis by a reductase enzyme encoded by *vrdA* gene outside the aflatoxin gene cluster

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ABSTRACT

Three reactions from hydroxyversicolorone to versicolorone, from versiconal hemiacetal acetate to versiconol acetate, and from versiconal to versiconol are involved in a metabolic grid in aflatoxin biosynthesis. This work demonstrated that the same reductase of *Aspergillus parasiticus* catalyzes the three reactions. The gene (named *vrdA*) encoding the reductase was cloned, and its sequence did not show homology to any regions in aflatoxin gene cluster. Its cDNA encoding a 38,566 Da protein was separated by three introns in the genome. Deletion of the *vrdA* gene in *A. parasiticus* caused a significant decrease in enzyme activity, but did not affect aflatoxin productivity of the fungi. Although the *vrdA* gene was expressed in culture conditions conducive to aflatoxin production, it was expressed even in the *aflR* deletion mutant. These results suggest that the *vrdA* is not an aflatoxin biosynthesis gene, although it actually participates in aflatoxin biosynthesis in cells.

1. Introduction

Aflatoxins are polyketide-derived secondary metabolites produced by certain strains of filamentous fungi, mainly *Aspergillus flavus* and *Aspergillus parasiticus* (Payne and Brown, 1998). Aflatoxins are highly toxic and carcinogenic in animals and humans, leading to hepatotoxicity, teratogenicity, immunotoxicity, and even death (Eaton and Groopman, 1994). Aflatoxin contamination of food and feed crops, such as wheat, corn, cotton, peanuts and tree nuts, is not only a serious health hazard but also an economic problem worldwide (Jelinek et al., 1989).

The biosynthetic pathway of aflatoxins has been extensively studied to develop strategies for reducing or eliminating aflatoxin contamination in food and feed. At least 18 enzymatic steps are required for conversion of acetyl coenzyme A (acetyl-CoA) to its final products, aflatoxins B_1, B_2, G_1 and G_2. Most of the enzymes have been identified, and the genes coding for the enzymes have been cloned and characterized (Minto and Townsend, 1997; Yabe and Nakajima, 2004; Yu et al., 2002). The genes constitute a large gene cluster encompassing 70 kb in the fungal genome, and their expression is positively regulated by the product of the regulatory gene, *aflR* (Payne et al., 1993; Price et al., 2006; Woloshuk and Prieto, 1998; Yu et al., 2004). The *aflR* gene product belongs to the family of zinc binuclear DNA-binding proteins, and deletion of the *aflR* gene represses the expression of the aflatoxin biosynthetic genes (Cary et al., 2000).

We previously reported that a metabolic grid among hydroxyversicolorone (HVN), versicolorone (VONE), versiconal hemiacetal acetate (VHA), versiconol acetate (VOAc), versiconol (VOH) and versiconal (VHOH) is involved in aflatoxin biosynthesis (Fig. 1), and that three kinds of enzymes (monooxygenase, esterase and reductase) function in the grid (Yabe et al., 1991a, 2003). In that study, HVN was converted to VHA by a cytosol monooxygenase, which requires NADPH as a cofactor (Yabe et al., 2003), and the *moxY* gene encoding the monooxygenase, which catalyzes the reaction from HVN to VHA, was identified (Wen et al., 2005). Involvement of the MoxY monooxygenase in the reaction from VONE to VOAc as well as HVN to VHA was shown by the deletion of the *moxY* gene causing the accumulation of both HVN and VONE (Wen et al., 2005). The resulting VHA and VOAc were converted to VHOH and VOH, respectively, by an esterase enzyme encoded by the *estA* gene (Chang et al., 2004). VHA also serves as a substrate for the reductase reaction from VHA to VOAc. Based on the similarities of partial structures of the substrates, the same reductase as

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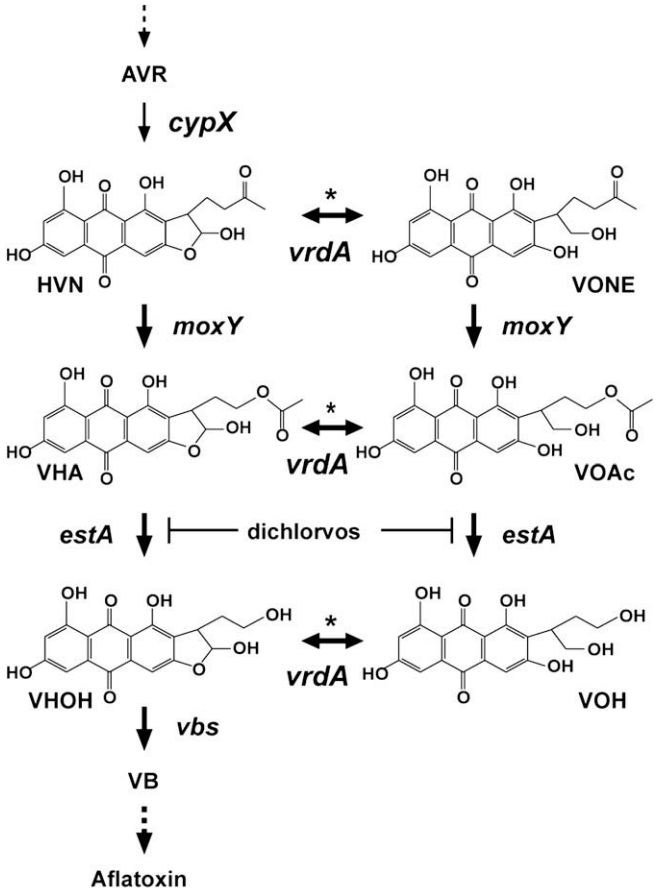

Fig. 1. Pathway for the metabolic grid from HVN to VHOH in aflatoxin biosynthesis. Steps catalyzed by VHA reductase encoded the *vrdA* gene. Asterisks indicate the VHA reductase reaction studied in detail in this work. Dichlorvos inhibits reactions from VHA to VHOH and from VOAc to VOH. Abbreviations: AVR, averufin; HVN, hydroxyversicolorone; VONE, versicolorone; VHA, versiconal hemiacetal acetate; VOAc, versiconol acetate; VHOH, versiconal; VOH, versiconol; VB, versicolorin B.

the VHA reductase is apparently involved in other reactions from HVN to VONE and from VHOH to VOH in the metabolic grid (Yabe et al., 1991a).

In the metabolic grid, the pathway from HVN to VHOH through VHA is likely the main pathway, because cell-free experiments revealed that these substances are produced mainly from averufin (Yabe et al., 2003). However, VONE, VOAc and VOH in the side pathway obviously operate, especially when a fungus culture becomes old or when a certain step in the pathway from HVN to VB is blocked (Chang et al., 2004; Wen et al., 2005; Yabe et al., 1991a). The reductase enzyme connects the main pathway and the side pathway, thus creating a metabolic grid for aflatoxin biosynthesis.

Aflatoxin production is dependent on the type of carbon source contained in the culture medium (Buchanan and Stahl, 1984; Davis and Diener, 1968). We previously reported that the reductase enzyme activity was significantly dependent on the aflatoxin-inducible carbon source, which was the same carbon source as that for the aflatoxin biosynthesis enzymes encoded by the genes in the aflatoxin gene cluster (Matsushima et al., 1994; Yabe et al., 1991a, 1989). The reductase enzyme activity also depended on the fungal species; the enzyme activity of *A. paraciticus* was much higher (about 33 times) than that of aflatoxin non-producing *Aspergillus oryzae* SYS-2 (IFO 4251) (Matsushima et al., 1994). These results suggested that the reductase enzyme would belong to the aflatoxin biosynthesis enzymes that are encoded in the aflatoxin gene cluster, although it is not included in the main pathway for aflatoxin biosynthesis.

We previously purified two reductase enzymes to homogeneity from the cytosol fraction of the mycelia of *A. parasiticus* NIAH-26 by monitoring the reaction from VHA to VOAc (Matsushima et al., 1994). Two enzymes (designated VHA reductases I and II) having the same enzyme activity were obtained, suggesting that these two enzymes are involved in aflatoxin biosynthesis in cells (Matsushima et al., 1994). However, in this study, we found that only the one enzyme corresponding to the larger enzyme (II) was reproducibly and successfully isolated from mycelia of the same strain, suggesting that another enzyme (reductase I) was a partial degradation product of the reductase II due to our previous poor techniques to inhibit certain contaminated proteases in crude fractions via many complicated purification steps in our early study (Matsushima et al., 1994). Therefore, our previous conclusion about involvement of two enzymes needed correction. This was accomplished in our current study, which demonstrated that a reductase enzyme corresponding to reductase II is the sole enzyme that catalyzes the reductase reactions in the metabolic grid in aflatoxin biosynthesis.

Our objective in this work was to determine the relationship between this reductase enzyme and aflatoxin biosynthesis. We isolated the genomic DNA as well as cDNA gene (named *vrdA*) encoding the reductase enzyme from *A. parasiticus*. Expression of the *vrdA* gene depended on culture conditions conducive to aflatoxin production, which was the same dependence seen for enzyme activity. However, this work demonstrated that the *vrdA* gene is not present within the aflatoxin gene cluster, and that the expression of the *vrdA* gene is not repressed by deletion of the *aflR* gene in *A. parasiticus*. These results strongly suggest that the reductase is not a common aflatoxin biosynthesis enzyme that is typically present in the aflatoxin gene cluster. This reductase might have a function other than aflatoxin biosynthesis, although it might inadvertently participate in aflatoxin biosynthesis because of its group substrate specificity to intermediates in aflatoxin biosynthesis.

2. Materials and methods

2.1. Fungal strains and culture conditions

A. parasiticus strains used in this study are listed in Table 1. SY liquid medium (6% sucrose and 2% yeast extract; aflatoxin-inducing medium) and PY liquid medium (4% peptone and 2% yeast extract; aflatoxin-non-inducing medium) were used for Northern analysis (Motomura et al., 1999). YES liquid medium (20% sucrose and 2% yeast extract; aflatoxin-inducing medium) and YEP liquid medium (20% peptone and 2% yeast extract; aflatoxin-non-inducing medium) were used for RT-PCR (Cai et al., 2008; Yan et al., 2004). GY medium (2% glucose and 0.5% glucose) was used as culture medium (Yabe et al., 1988).

2.2. DNA and RNA isolation

Genomic DNA was purified from *A. parasiticus* NIAH-26 as described previously (Motomura et al., 1999). Genomic DNA was also purified from *A. parasiticus* SYS-4 and the *vrdA* deletion mutant by using Nucleon PhytoPure (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

For cloning, mycelia of *A. parasiticus* NIAH-26 was cultured in YES liquid medium at 28 °C for 4 days, and then total RNA was extracted from the mycelia as described previously (Motomura et al., 1999). mRNA was purified by an oligo(dT)-cellulose column using an mRNA purification kit (GE Healthcare) from total RNA. For