Stereochernistry during Aflatoxin Biosynthesis: Cyclase Reaction in the Conversion of Versiconal to Versicolorin B and Racemization of Versiconal Hemiacetal Acetate

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(1'R,2'S)-(−)-aflatoxins are produced from racemic versiconal hemiacetal acetate (VHA) through complicated pathways, including a metabolic grid involving VHA, versiconal acetate (VOAc), versicolon, and versiconal (VHOH), and a reaction sequence from VHOH to versicolorin A (VA) through (−)-versiconol B (VB) (18) and (−)-versicolorin C (1, 18, 28). Also, (VC) (or VB) (19). Aspergillus parasiticus NIAH-26, both (2'S)- and (2'R)-VOAc enantiomers were formed at about a 1:2 ratio from racemic VA in the presence of NADPH and dichlorvos (dimethyl 2,2-dichlorovinylphosphate). Also, the esterase activity catalyzing the conversion of VA to VHOH or of VOAc to versicolon did not show the stereosepecificity for the 2' carbon atom of VA or VOAc. However, when racemic VA or racemic VHOH was incubated with the esterase, (1'R,2'S)-(−)-VB was formed exclusively. Furthermore, only (1'R,2'S)-(−)-VB, and not (1'S,2'R)-(−) antipode, served as a substrate for desaturase activity in the microsome fraction catalyzing the conversion of VB to VA. These results demonstrate that the stereosepecificity of bis-furan moiety in aflatoxin molecules is determined by the cyclase enzyme catalyzing the reaction from VHOH to VB, and the (1'R,2'S)-(−) configuration was further confirmed by the subsequent desaturase reaction. Remarkably, we found nonenzymatic racemization in both the (2'R)- and (2'S)-VA enantiomers, and it was dependent upon the temperature and alkaline conditions.

MATERIALS AND METHODS

Microorganism. A. parasiticus NIAH-26 was used (29). NIAH-9, a VA-accululating mutant which does not produce aflatoxins, was used in the preparation of VA and VOAc (28).

Standard samples of metabolites. Racemic VA and racemic VOAc (Fig. 1) were prepared from mycelia of the mutant strain NIAH-9, which had been cultured in YES (2% yeast extract, 20% sucrose) medium supplemented with 100 ppm (0.14 mg/ml) dichlorvos (dimethyl 2,2-dichlorovinylphosphate), which is an inhibitor of esterase (21, 30). (1'R,2'S)-(−)-VA, (1'R,2'S)-(−)-VB, (−)-VC, and (−)-VHOH were prepared from mycelia of A. versicolor (Vuillemin) Tiroboshi (13, 14, 16). In the preparation of VA enantiomers, a standard sample of racemic VA was first separated by
using a Chiralcel OD column (described below), and then the peak fraction of each enantiomer was pooled, dried, and solubilized with methanol. The VOAc enantiomer was prepared by separating a standard sample of racemic VOAc by using a Chiralcel OJ column (described below). According to the order of elution from the column, the enantiomers of the metabolites are denoted by the subscripts 1 and 2. (2'R)-VC and (2'S)-VC (= VB) were prepared from the (2'R)- and (2'S)-VHOH enantiomers, respectively, by spontaneous de-

hydration. Each VHOH enantiomer was prepared by separating the racemic VHOH by using a Chiralcel OD column after 131 μM VHA was incubated with porcine liver esterase (0.15 mg/ml; Boehringer-Mannheim Co. Ltd.) in solution A (0.1 M potassium phosphate buffer [pH 7.5], 1% glycerol). The concentration of the metabolites in methanol was determined from UV absorption spectra by using molar absorption coefficients (2, 6, 13, 16, 25) as follows (in M⁻¹ cm⁻¹): VHA (480 nm), 7,250; VOAc (453 nm), 8,500; VOH (455 nm), 7,400; VA (452 nm), 8,166; VB (450 nm), 8,700; VC (450 nm), 10,700.

**Cell-free studies.** The purified cytosol protein fraction and the microsome fraction were prepared from the mycelia of NIAH-26 (29). The enzyme assay and chiral HPLC analysis were done as described in the accompanying article (29), unless otherwise stated. The concentration of the substrate was 60 μM, and the reaction was terminated by adding 80 μl of ethyl acetate instead of water-saturated ethyl acetate. The ethyl acetate extract was always dried by keeping the vessel open to the atmosphere under darkness. In the HPLC analyses, a Chiralcel OJ or OD column (0.46 by 25 cm; Daisel Chemical Industry, Ltd., Tokyo, Japan) was used.

To investigate the stereospecificity of the VHA dehydrogenase activity, the cytosol fraction was incubated with VHA, NADPH, and 0.14 mg of dichlororphenol per ml and the reaction products were analyzed by using a Chiralcel OJ or OD column with a solution of n-hexane-ethanol-trifluoroacetic acid (n-hexane-ethanol-TFA; OD column, 85:15:0.2, vol/vol/vol; OD column, 90:10:0.2, vol/vol/vol). For the esterase activity catalyzing the conversion of VOAc to VOH, the cytosol fraction or porcine liver esterase (0.17 mg/ml) was incubated in solution A with VOAc added. After the reaction was terminated by adding 3 μl of formic acid, the mixture was centrifuged, and then 20 μl of the supernatant was analyzed by using a silica gel thin-layer chromatography plate (Silia Gel 60, no. 5721; Merck & Co., Inc., Rahway, N. J.) and a developing solution containing chloroform-ethyl acetate-90% formic acid (6:3:1, vol/vol/vol). After the pigments of the parts corresponding to the Rf values for VOAc and VHOH on the thin-layer chromatography plate were extracted with ethyl acetate, dried, and then solubilized with methanol, each pigment was analyzed by using a Chiralcel OJ column with an n-hexane-ethanol-TFA (87:13:0.2, vol/vol/vol) solution. For the conversion of VHA to VB, the cytosol fraction was incubated with VHA, and then the reaction products were analyzed by using a Chiralcel OD column with an n-hexane-ethanol-TFA (90:10:0.2, vol/vol/vol) solution. For the cyclase activity in the conversion of VHOH to VB, racemic VHOH was first prepared from VHA by using porcine liver esterase. Porcine esterase (0.17 mg/ml) was incubated with 60 μM VHA at 37°C in solution A for 60 min. The reaction was then terminated by adding 80 μl of ethyl acetate and mixing. Sixty microliters of the resultant ethyl acetate layer was then transferred to a new tube and dried. After the residue was solubilized with solution A by using a Vortex mixer, a second conversion of the resultant VHOH to VB was started by adding the cytosol fraction (with a final concentration of 0.2 mg/ml), making the total volume of the mixture 50 μl. The reaction products were analyzed by using a Chiralcel OD column with an n-hexane-ethanol-TFA (90:10:0.2, vol/vol/vol) solution. For the desaturase conversion of VB to VA, the microsome fraction (2.4 mg/ml) was incubated with 15 μM VC enantiomer (either type) in solution A at 30°C for 60 min, and the reaction products were analyzed by using a Chiralcel OJ column with an n-hexane-ethanol-TFA (85:15:0.2, vol/vol/
vol) solution. To calculate the amount of the metabolites, the standard curve for VB was used.

**Racemization of VHA.** Racemization was started by adding 14 μM VHA enantiomer (either type) to either a solution containing 40 μM potassium phosphate (pH 7.5) and 10% glycerol or distilled water (Milli Q, Millipore Corp.). The reaction was carried out at either 37 or 4°C (on ice) for various times and then terminated by adding 80 μl of ethyl acetate. The resultant products were analyzed by using a Chiralcel OD column with an n-hexane–ethanol–TFA (90:10: 0.2, vol/vol/vol) solution. The pH profile of the rate of racemization from VHA₁ to VHA₂ was performed at 37°C for 15 min by using a series of 100 mM potassium phosphate buffers with different pH levels, namely, pH 6.0, 6.5, 7.0, 7.5, and 8.0.

**Determination of the configuration of the metabolites.** Since the VC₂ peak on the Chiralcel OD or OJ chromatograms corresponded to that of (−)-VB, we assumed that VC₁ and VC₂ were (1'R,2'S)-(+) and (1'R,2'S)-(−)-VC (=VB) enantiomers, respectively. To determine the configurations of the VHA enantiomers on a Chiralcel OD chromatogram, each VHA enantiomer was mixed in a 11.6 M HCl solution with a Vortex mixer for 2 min at room temperature, and then the products were examined by using a Chiralcel OD column. Since VHA₁ and VHA₂ were converted to VC₁ and VC₂ (=VB), respectively, by this acid treatment, we concluded that VHA₁ and VHA₂ have a (2'R) and (2'S) configuration, respectively. Also, we determined that VHOH₁ and VHOH₂ have (2'R) and (2'S) configurations, respectively, because VHOH₁ and VHOH₂ spontaneously changed to VC₁ and VC₂, respectively.

On the other hand, when each VHA enantiomer was incubated with the cytosol fraction in the presence of dichlorvos and NADPH, VOAc₁ and VOAc₂ were mainly formed from VHA₂ and VHA₁, respectively. Therefore, we determined that VOAc₁ and VOAc₂ have (2'S) and (2'R) configurations, respectively. Also, in similar cell-free experiments using the cytosol fraction, VOAc₁ and VOAc₂ were converted to VHOH₁ and VHOH₂, respectively. We concluded that VOH₁ and VOH₂ were (2'S) and (2'R) enantiomers, respectively. The (−)-VOH standard sample corresponded to VOH₁ on a Chiralcel OJ chromatogram.

**RESULTS**

Both VHA enantiomers served as substrates for the VHA dehydrogenase conversion of VHA to VOAc. Chiralcel OD and OJ columns with ternary systems consisting of n-hexane–ethanol–TFA effected the separation of racemic VHA and VOAc, respectively (Fig. 2A). By using these conditions, we found that VHA and VOAc standard samples prepared from VA-accumulating mutant NIAH-9 were racemates composed of equal amounts of enantiomers.

When the cytosol fraction was incubated with the standard sample of VHA in the presence of NADPH and dichlorvos for various times, both VOAc enantiomers were produced. The reaction continued during the 60 min of incubation, with the amount of (2'R)-VOAc₂ reaching about twice that of VOAc₂ (2'S) as shown in Fig. 2B. This result indicates that the VHA dehydrogenase preferred (2'R)-VHA to (2'S)-antipode as a substrate. However, unexpectedly, the amount of (2'R)-VHA₁ enantiomer remaining in the reaction mixture decreased almost to the same extent as the amount of (2'S)-VHA₂ enantiomer did. Therefore, the ratio of the total amount of metabolites (VHA₁, VOAc₂) having a (2'R) configuration to those (VHA₂, VOAc₁) having a (2'S) configuration reached about 2:1 after the reaction, while the total amount of the resultant VOAc enantiomers almost equaled that of the VHA consumed.

Both VOAc enantiomers served as substrates for the esterase conversion of VOAc to VOH. The enantiomers of VOAc or VOH were resolved by using a Chiralcel OJ column (Fig. 3A and B). When the cytosol fraction was incubated with the racemic VOAc sample, similar amounts of (2'S)-VOH₁ and (2'R)-VOH₂ were produced from racemic VOAc, although the cytosol enzyme weakly preferred (2'S)-VOAc₂ to (2'R)-VOAc₂ (Fig. 3C). When porcine esterase was incubated with VOAc, similar results were obtained (data not shown).

**Stereospecific formation of VB from racemic VHA and racemic VHOH.** When the cytosol fraction was incubated with racemic VHA for 20 min, (1'S,2'R)-VC₁, (1'R,2'S)-VC₂ (=VB), and two other kinds of substances were formed (Fig. 4A). The peaks of these last two substances corresponded to those of (2'R)-VHOH₁ and (2'S)-VHOH₂ that had been prepared from VHA by using porcine esterase (data not shown). Even though the retention times of the VHOH enantiomers partially overlapped the retention times of
VHA, transient formation of VHOH$_1$ and VHOH$_2$ was apparent.

Figure 4B shows the quantitative relationship of each substance during the conversion. The amount of $\text{(1'}R,2'S)\text{-VC}_2$ (=VB) monotonically increased, peaking at 90% of the total VHA amount initially added. In contrast, the amount of $\text{(1}S,2'R)\text{-VC}_1$ did not change after a slight amount of VC$_1$ was formed near $t = 0$, and the amount of both remaining VHA enantiomers monotonically decreased. It is apparent that most of both enantiomers of VHA or VHOH was converted to $\text{(1}R,2'S)\text{-VC}_2$ (=VB).

To show the dominant formation of VB from VHA in more detail, we investigated the cyclase conversion of racemic VHOH to VB (Fig. 5). When the cytosol fraction was incubated with racemic VHOH that had been prepared from VHA by porcine esterase, $\text{(1'}R,2'S)\text{-VC}_3$ (=VB) increased monotonically and peaked at 90% of the amount of VHA initially added to the reaction mixture, whereas $\text{(1}S,2'R)\text{-VC}_1$ never formed. Both enantiomers of VHOH decreased with time, with a corresponding production of VC$_2$ (=VB). When either VC$_1$ or VC$_2$ (=VB) enantiomer was incubated with the cytosol fraction, neither the conversion from VC$_1$ to VC$_2$ nor the reverse occurred (data not shown).

Nonenzymatic racemization of VHA. When the (2'R)\text{-VHA} enantiomer was incubated in the reaction mixture at 37°C for various times and without the cytosol fraction, (2'S)\text{-VHA}$_2$ was formed and 45% of the VHA$_2$ was converted to VHA$_2$ after 60 min of incubation (Fig. 6). Also, when the (2'S)\text{-VHA}$_2$ isomer was incubated under the same conditions, 46% of this substance was converted to (2'R)\text{-VHA}$_2$ after 60 min of incubation (data not shown). When incubation was carried out at 4°C, racemization of VHA was not detected. In addition, racemization seldom occurred when Milli Q water instead of the reaction mixture was used, even at 37°C. When the racemic standard sample of VHA was incubated under the same conditions, the ratio of the concentrations of both enantiomers did not change (data not shown).

Racemization from (2'R)\text{-VHA}$_1$ to (2'S)\text{-VHA}$_2$ was dependent upon the pH of the incubation solution (Fig. 7). In acidic solutions, racemization scarcely occurred; however, the rate of the racemization increased with increasing pH. At a pH of 8.0, about 40% of the VHA$_1$ was converted to VHA$_2$ after 15 min of incubation.

Sterespecific desaturation conversion of VB to VA. VC enantiomers and VA could be separated by using a Chiralcel OD column (Fig. 8). When (1'S,2'R)\text{-VC}$_2$ was incubated with the microsome fraction in the absence of NADPH for 60 min, VA was not formed. Although a slight amount of VA was formed in the presence of NADPH, this VA was probably produced from a (2'S)\text{-VC}$_2$ contaminant in the sample of VC$_1$. Also, VA was not formed from VC$_2$ (=VB) in the absence of NADPH. In contrast, in the presence of NADPH, a significant amount of VA was formed with a corresponding decrease of (1'R,2'S)\text{-VC}$_3$. We therefore concluded that only (1'R,2'S)-VB was converted to (1'R,2'S)-VA. Furthermore, when racemic VC was incubated with the microsome fraction, only the amount of the VC$_2$ (=VB)
FIG. 5. Time course of the cyclase reaction from racemic VHOH to (1'R,2'S)-VC2 (=VB). The cytosol was incubated with racemic VHOH, which had been prepared from VHA by porcine esterase. The reaction products were then analyzed by using a Chiralcel OD column. Symbols: O, (2'R)-VHOH1; ■, (2'S)-VHOH2; □, (2'R)-VC1; ●, (2'S)-VC2 (=VB).

component decreased, with a corresponding formation of VA, whereas the amount of the VC1 enantiomer in the VC sample did not change (data not shown).

DISCUSSION

As described in the accompanying article (29), the use of chiral HPLC columns made it possible for us to examine the configurational changes of metabolites. By using chiral OD and OJ columns, we were able to specify the determining step in the configuration of aflatoxins during their biosynthe-

FIG. 6. Progress of the racemization of a VHA enantiomer under different incubation conditions. (2'R)-VHA1 was incubated in a solution containing 40 µM potassium phosphate (pH 7.5) and 10% glycerol at 37°C (●) or 4°C (■) or in distilled water at 37°C (▲) for various intervals. The amount of VHA enantiomer was then measured by using a Chiralcel OD column. Symbols: ●, ■, and ▲, (2'S)-VHA2 enantiomer; □, total amount of VHA isomers.

sis. The proposed metabolic scheme for the conversion of VHA to VA is summarized in Fig. 9.

We recently suggested on the basis of the similarity in the structures of VOAc and VHA and of VOH and VHOH that the same esterase may catalyze a conversion of VOAc to VOH (28). In the current study, we showed that both (2'R)

FIG. 7. The pH dependency of racemization of VHA1 to VHA2. VHA1 (10 µM) was incubated at 37°C for 15 min in a 100 mM potassium phosphate buffer (from pH 6.0 to 8.0). The amount of VHA enantiomer was then measured by using a Chiralcel OD column with n-hexane–ethanol–TFA (86:14:0.2, vol/vol/vol). The percentage of the resultant (2'S)-VHA2 relative to the total VHA (215 ± 12 pmol) is shown. The dashed line indicates the percentage of (2'S)-VHA2 before the reaction.

FIG. 8. Stereospecific conversion of VC2 (=VB) to VA. The microsome fraction was incubated with VC1 (A and B) or VC2 (C and D) for 60 min in the absence (A and C) or presence (B and D) of NADPH and then analyzed by using a Chiralcel OJ column.
and (2'S) enantiomers of VHA and VOAc serve as substrates for the esterase prepared from the mold as well as the porcine esterase (Fig. 3 and 4). These results indicate that the esterase is not involved in the determination of the stereostructure of bis-furan moiety of aflatoxins. Interestingly, it was reported that the VOH standard sample prepared from A. versicolor was one stereoisomer having (−) optical rotation (16), although this study shows that (2'R)- and (2'S)-VOH enantiomers were produced from racemic VOAc by using a cell-free system of A. parasiticus (Fig. 3). These results may reflect the differences in mold species.

As for VHA dehydrogenase activity, both (2'R)- and (2'S)-VOAc were produced from racemic VHA (Fig. 2). We recently suggested on the basis of the similarity in the structures of VOAc and VOH and of VHA and VHOH that the same dehydrogenase(s) may catalyze a conversion of VOH to VHOH (28). Therefore, the reactions between VHA and VOAc or VOAc and VHOH and VOH may not be related to the determination of the stereospecificity of aflatoxins. However, this enzyme preferred the (2'R)-VHA isomer to the (2'S) antipode at about a 2:1 ratio. Although this might reflect the partial stereospecificity of this enzyme to this conformation, we cannot neglect the possibility that several different dehydrogenase molecules might be involved in this reaction, because we recently found that the VHA dehydrogenase activity in the cytosol formed two peaks on DEAE anion-exchange chromatography analysis (26a).

VHOH seemed to be in equilibrium with respect to its four stereoconfigurations, (1'R,2'S), (1'S,2'S), (1'R,2'R), and (1'S,2'R) (11). The former two enantiomers [having (2'S) configurations] can be directly dehydrated to (1'R,2'S)-VB because the 1'-hemiacetal moiety undergoes rapid inversion of configuration. In contrast, direct conversion of the latter enantiomers [having (2'R) configurations] to (1'R,2'S)-VB is stereochemically impossible. In fact, (2'R)-VHOH was nonenzymatically converted to (1'S,2'R)-(+)-VC1, but not to (2'S)-VC2 (= VB), during the isolation procedure of this substance in this study. However, we showed that (2'R)-VHOH, as well as the (2'S) enantiomer, was also converted to (1'R,2'S)-(−)-VB by the cyclase reaction (Fig. 5). This discrepancy was rationalized by the observation that both enantiomers of VHA were nonenzymatically racemized in the reaction mixture, approaching a mixture composed of equal amounts of (2'R) and (2'S) enantiomers (Fig. 6). Since VHOH has the same hemiacetal structure as VHA, nonenzymatic racemization of VHOH likely occurs in VHOH molecules under similar conditions. Therefore, in the cell-free system, two independent reactions may occur at the same time, the stereospecific conversion of (2'S)-VHOH to (1'R,2'S)-(−)-VB by the cyclase enzyme and the nonenzymatic racemization from the remaining (2'R)-VHOH to (2'S) antipode. The conclusion that racemization between (2'R) and (2'S) enantiomers of VHA or VHOH occurs was also supported by other experimental results. For example, almost the same amount of enantiomers of VHA remained during the conversion of VHA to VOAc in spite of the predominant formation of VOAc2 (Fig. 2); also, (2'R)-VHA, as well as its antipode, was converted to (1'R,2'S)-(−)-VB (Fig. 4). Therefore, the configurations of aflatoxins are determined by the cyclase enzyme catalyzing the conversion of (2'S)-VHOH to (1'R,2'S)-(−)-VB.

The scheme proposed for the racemization of VHA or VHOH is shown in Fig. 10. The transitory formation of the open form of VHA or VOH that has one aldehyde group in various polar solutions has been reported (11, 25, 26). Also, another hemiacetal substance, ABF1, hemiacetal, was reported to undergo structural changes to form a phenolate ion with two aldehyde groups (3, 4, 20). In these minor open forms of hemiacetal substances, a single asymmetric center apparently exists next to an aldehyde group. Therefore, it should be possible for the optically active compound to be racemized by tautomerism. In the current study, we showed that the racemization of VHA was dependent upon the reaction temperature and basic condition of the reaction solution (Fig. 6 and 7). Temperature and pH dependency of the reaction is generally observed in tautomerism. In a basic solution, the position of 2' on the substances may be deprotonated and consequently racemized through keto-enol tautomerism. This scheme is supported by another report that showed that the optical rotation of ABF1 hemiacetal in a basic solution approached zero within minutes (4).

Although this current study showed that (1'R,2'S)-VB is exclusively produced from VHOH, VC, instead of VB, has generally been isolated from various molds (8, 17). We found that VHOH was very unstable and easily converted to VC during the experiments, especially under acidic conditions. Most of the VHOH was nonenzymatically converted to VC when water-saturated ethyl acetate, which was a weakly
With a VROL, to verted pure ethyl acetate to versiconal and versicolorin C in extracts from Aspergillus parasiticus. Mycopathologia 110:31-35.


conal acetate, versiconal acetate, and versiconol, metabolites from cultures of Aspergillus parasiticus treated with dichlorvos.
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