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Stereochemistry 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, versiconol acetate (VOAc), versiconol, and versiconal (VHOH), and a reaction sequence from VHOH to versicolorin A (VA) through (−)-versicolorin B (VB) [or (±)-versicolorin C] (K. Yabe, Y. Ando, and Y. Hamasaki, J. Gen. Microbiol. 137:2469–2475, 1991; K. Yabe, Y. Ando, and T. Hamasaki, Agric. Biol. Chem. 55:1907–1911, 1991). In this study, we examined stereochemical changes of substances formed during the conversion of VHA to VA by using chiral high-performance liquid chromatography. In cell-free experiments using the cytosol of *Aspergillus parasiticus* NIAH-26, both (2′*S*)- and (2′*R*)-VOAc enantiomers were formed at about a 1:2 ratio from racemic VHA in the presence of NADPH and dichlorvos (dimethyl 2,2-dichlorovinylphosphate). Also, the esterase activity catalyzing the conversion of VHA to VHOH or of VOAc to versiconol did not show the stereospecificity for the 2′ carbon atom of VHA or VOAc. However, when racemic VHA or racemic VHOH was incubated with the cytosol, (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 stereoconfiguration 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*)-VHA enantiomers, and it was dependent upon the temperature and alkaline conditions.

Aflatoxins B_1 (AFB_1), G_1 (AFG_1), B_2 (AFB_2), and G_2 (AFG_2) are major, naturally occurring aflatoxins that are produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*. The biosynthetic pathway of aflatoxins, summarized in Fig. 1, has been extensively studied (see references in the accompanying article [29]). Our research group, among others, reported the enzymatic conversion from versiconal hemiacetal acetate (VHA) to versicolorin C (VC) (or versicolorin B [VB]) via the formation of versiconal (VHOH) (1, 18, 28). Also, we recently reported that a metabolic grid of VHA, versiconol acetate (VOAc), versiconol (VOH), and VHOH, which is catalyzed by dehydrogenase and esterase, is involved in aflatoxin biosynthesis (28) and that VB and VC were converted to versicolorin A (VA) by a desaturase enzyme (27).

AFB_1 (22, 23), AFG_1 (5), AFB_2 (24), and sterigmatocystin (10) have the same absolute configuration [i.e., (1′*R*,2′*S*)] on bis-furan moieties of the molecules. This same configuration is suggested for VA (12, 14), VB (14), dihydrosterigmatocystin (15), and dihydrodemethylsterigmatocystin (15) from the results of various spectroscopic methods. VOH prepared from *Aspergillus versicolor* is also an optically active substance (16). In contrast, VC is a racemate of VB and is composed of (1′*S*,2′*R*) and (1′*R*, 2′*S*) enantiomers (9). VC, instead of VB, has been generally isolated as a metabolite of *A. parasiticus* and *A. versicolor* molds (8, 17), and aflatoxins have been produced from VC as well as VB in feeding experiments (27). Lin and Anderson recently suggested that VB is predominantly produced from VHOH in a cell-free system with pure cyclase enzyme catalyzing the conversion of VHOH to VC (or VB) (19). VHA is also a racemate composed of (2′*S*) and (2′*R*) enantiomers (7). On the basis of these facts, we speculated that the stereoconfiguration of aflatoxins in their bis-furan moiety can be determined at any step during the conversion of VHA to VA.

To ascertain the step that determines the configuration of the bis-furan structure of aflatoxins, we examined the configurations of the reaction products during the conversion of VHA to VA in a cell-free system by using chiral high-performance liquid chromatography (chiral HPLC). This study was also the first to determine nonenzymatic racemization of VHA and VHOH.

MATERIALS AND METHODS

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

Standard samples of metabolites. Racemic VHA 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 (−)-VOH were prepared from mycelia of *A. versicolor* (Vuillemin) Tiraboschi (13, 14, 16). In the preparation of VHA enantiomers, a standard sample of racemic VHA was first separated by

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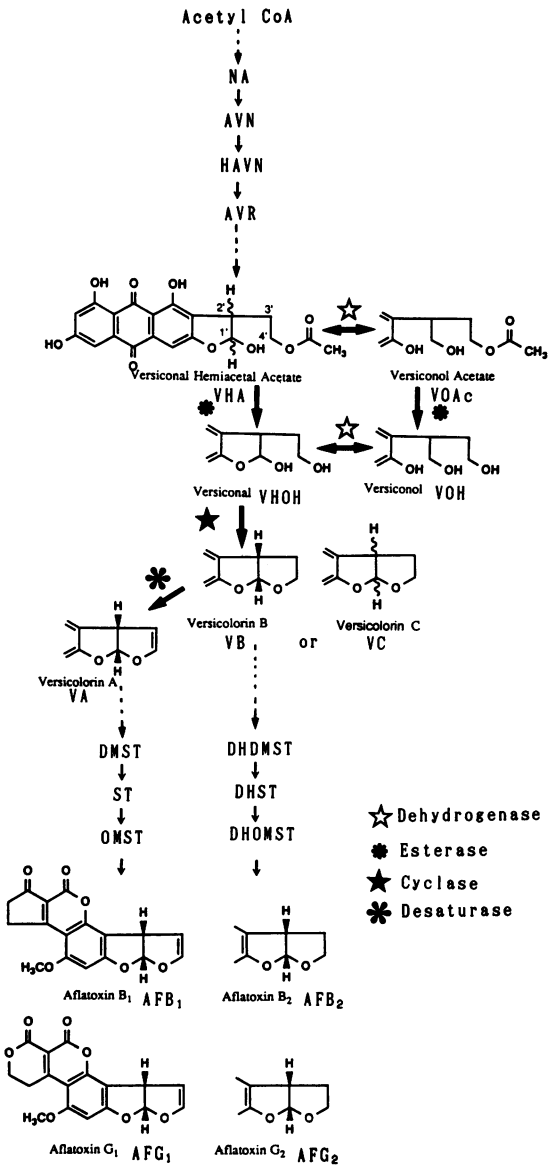

FIG. 1. Metabolic scheme for aflatoxin biosynthesis showing structures of the substances used in this study. The bis-furan structures of aflatoxins show a (1′*R*,2′*S*) configuration. Solid arrows, confirmed reactions; dashed arrows, unconfirmed reactions. Enzymes involved in the pathway from VHA to VA are indicated by various symbols. Abbreviations: acetyl CoA, acetyl coenzyme A; NA, norsolorinic acid; AVN, averantin; HAVN, 5′-hydroxyaverantin; AVR, averufin; DMST, demethylsterigmatocystin; ST, sterigmatocystin; OMST, *O*-methylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; DHST, dihydrosterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin.

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_1 and (2′*S*)-VC_2 (=VB) were prepared from the (2′*R*)- and (2′*S*)-VHOH enantiomers, respectively, by spontaneous dehydration. 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 (90 mM potassium phosphate buffer [pH 7.5], 10% 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 dichlorvos 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; OJ 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 (Silica 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 R_f 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 VHA_1 to VHA_2 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 VC_2 peak on the Chiralcel OD or OJ chromatograms corresponded to that of (−)-VB, we assumed that VC_1 and VC_2 were (1′*S*,2′*R*)-(+)- 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 VHA_1 and VHA_2 were converted to VC_1 and VC_2 (=VB), respectively, by this acid treatment, we concluded that VHA_1 and VHA_2 have a (2′*R*) and (2′*S*) configuration, respectively. Also, we determined that $VHOH_1$ and $VHOH_2$ have (2′*R*) and (2′*S*) configurations, respectively, because $VHOH_1$ and $VHOH_2$ spontaneously changed to VC_1 and VC_2, respectively.

On the other hand, when each VHA enantiomer was incubated with the cytosol fraction in the presence of dichlorvos and NADPH, $VOAc_1$ and $VOAc_2$ were mainly formed from VHA_2 and VHA_1, respectively. Therefore, we determined that $VOAc_1$ and $VOAc_2$ have (2′*S*) and (2′*R*) configurations, respectively. Also, in similar cell-free experiments using the cytosol fraction, $VOAc_1$ and $VOAc_2$ were converted to VOH_1 and VOH_2, respectively. We concluded that VOH_1 and VOH_2 were (2′*S*) and (2′*R*) enantiomers, respectively. The (−)-VOH standard sample corresponded to VOH_1 on a Chiralcel OJ chromatogram.

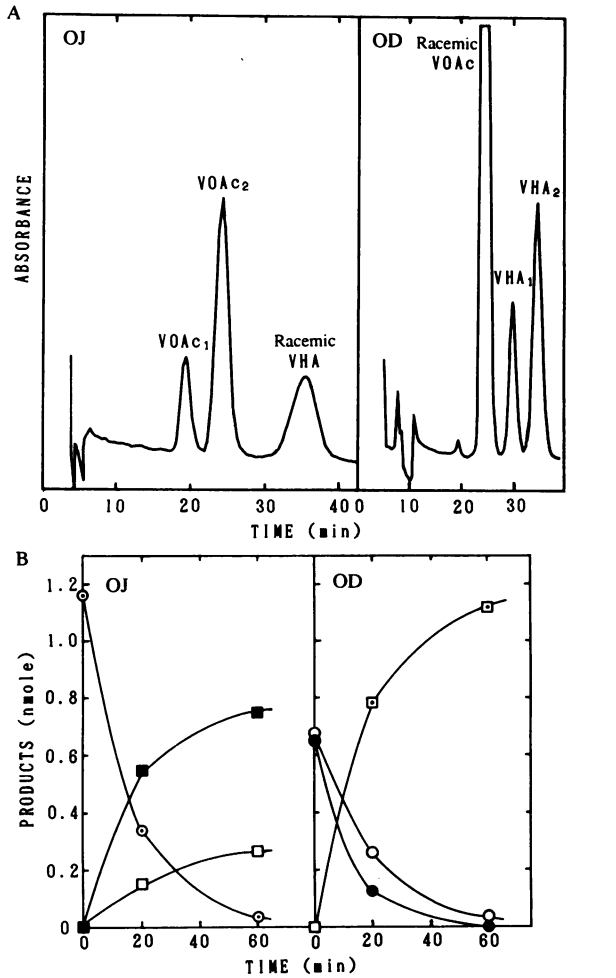

FIG. 2. (A) Chiral HPLC separation of the reaction products from VHA for 20 min by VHA dehydrogenase activity by using either a Chiralcel OJ or OD column; (B) time course of the amount of VHA and VOAc in the reaction mixture examined. Symbols: □, (2′*S*)-$VOAc_1$; ■, (2′*R*)-$VOAc_2$; ●, (2′*R*)-VHA_1; ○, (2′*S*)-VHA_2; ⊙, racemic VHA; ⊡, racemic VOAc.

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*)-$VOAc_2$ reaching about twice that of $VOAc_1$ (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*)-VHA_1 enantiomer remaining in the reaction mixture decreased almost to the same extent as the amount of (2′*S*)-VHA_2 enantiomer did. Therefore, the ratio of the total amount of metabolites (VHA_1, $VOAc_2$) having a (2′*R*) configuration to those (VHA_2, $VOAc_1$) 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*)-VOH_1 and (2′*R*)-VOH_2 were produced from racemic VOAc, although the cytosol enzyme weakly preferred (2′*S*)-$VOAc_1$ to (2′*R*)-$VOAc_2$ (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*)-VC_1, (1′*R*,2′*S*)-VC_2 (=VB), and two other kinds of substances were formed (Fig. 4A). The peaks of these last two substances corresponded to those of (2′*R*)-$VHOH_1$ and (2′*S*)-$VHOH_2$ 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

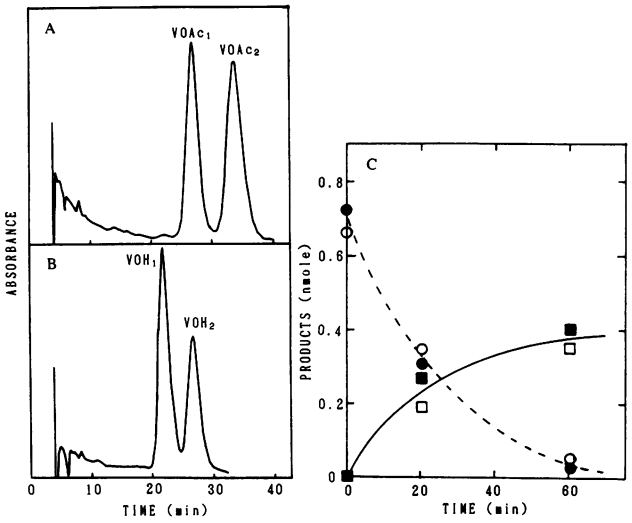

FIG. 3. Chiral HPLC separation of the reaction products from VOAc for 20 min by esterase activity in which the remaining VOAc (A) and the resultant VOH (B) were isolated by thin-layer chromatography and then analyzed by using a Chiralcel OJ column. The time course of the amount of VOAc and VOH is shown in panel C. Symbols: ●, (2′*S*)-$VOAc_1$; ○, (2′*R*)-$VOAc_2$; ■, (2′*S*)-VOH_1; □, (2′*R*)-VOH_2.

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 (1′*R*,2′*S*)-VC_2 (=VB) monotonically increased, peaking at 90% of the total VHA amount initially added. In contrast, the amount of (1′*S*,2′*R*)-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 (1′*R*,2′*S*)-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, (1′*R*,2′*S*)-VC_2 (=VB) increased monotonically and peaked at 90% of the amount of VHA initially added to the reaction mixture, whereas (1′*S*,2′*R*)-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*)-VHA_1 enantiomer was incubated in the reaction mixture at 37°C for various times and without the cytosol fraction, (2′*S*)-VHA_2 was formed and 45% of the VHA_1 was converted to VHA_2 after 60 min of incubation (Fig. 6). Also, when the (2′*S*)-VHA_2 isomer was incubated under the same conditions, 46% of this substance was converted to (2′*R*)-VHA_1 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

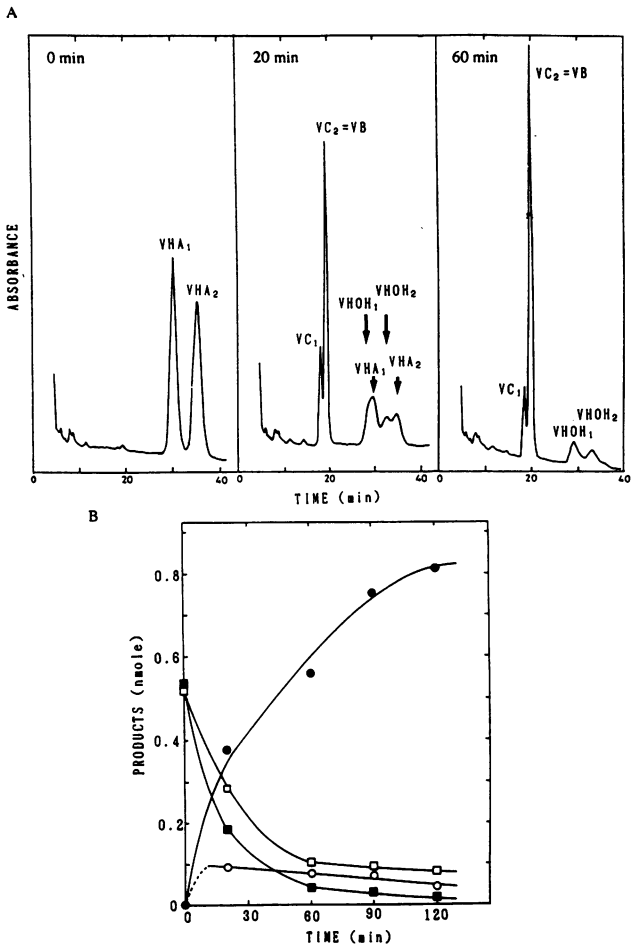

FIG. 4. (A) Chiral HPLC separation of the reaction products from VHA for various incubation times by esterase and cyclase activities by using a Chiralcel OD column; (B) time course of the amount of (2′*R*)-VC_1 (○), (2′*S*)-VC_2 (=VB) (●), sum of (2′*R*)-VHA_1 and -$VHOH_1$ (□), and sum of (2′*S*)-VHA_2 and -$VHOH_2$ (■).

concentrations of both enantiomers did not change (data not shown).

Racemization from (2′*R*)-VHA_1 to (2′*S*)-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.

Stereospecific desaturation conversion of VB to VA. VC enantiomers and VA could be separated by using a Chiralcel OJ column (Fig. 8). When (1′*S*,2′*R*)-VC_1 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*)-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*)-VC_2. 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)

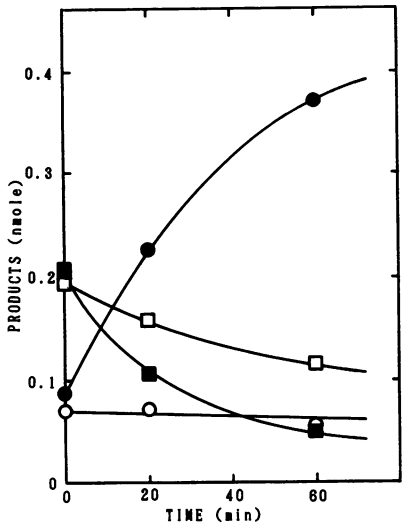

FIG. 5. Time course of the cyclase reaction from racemic VHOH to (1'*R*,2'*S*)-VC_2 (=VB). The cytosol was incubated with racemic VHOH, which had been prepared from VHA by porcine esterase. The reaction products were then analyzed by using a Chiralcel OD column. Symbols: □, (2'*R*)-$VHOH_1$; ■, (2'*S*)-$VHOH_2$; ○, (2'*R*)-VC_1; ●, (2'*S*)-VC_2 (=VB).

component decreased, with a corresponding formation of VA, whereas the amount of the VC_1 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 biosynthesis. 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*)

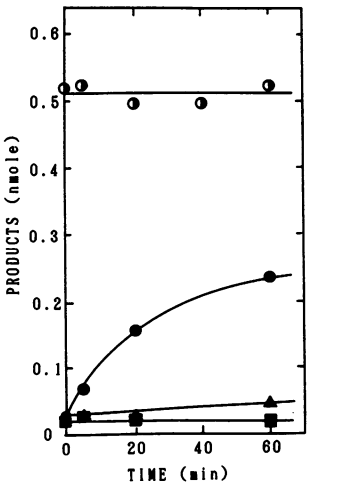

FIG. 6. Progress of the racemization of a VHA enantiomer under different incubation conditions. (2'*R*)-VHA_1 was incubated in a solution containing 40 μM potassium phosphate (pH 7.5) and 10% glycerol at 37°C (●) or 4°C (■) or in distilled water at 37°C (▲) for various intervals. The amount of VHA enantiomer was then measured by using a Chiralcel OD column. Symbols: ●, ■, and ▲, (2'*S*)-VHA_2 enantiomer; ◑, total amount of VHA isomers.

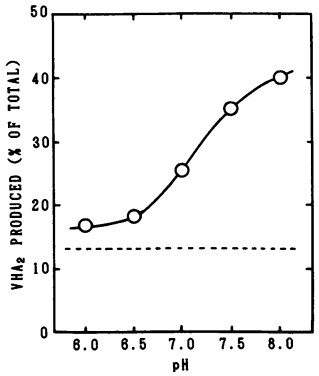

FIG. 7. The pH dependency of racemization of VHA_1 to VHA_2. VHA_1 (10 μM) was incubated at 37°C for 15 min in a 100 mM potassium phosphate buffer (from pH 6.0 to 8.0). The amount of VHA enantiomer was then measured by using a Chiralcel OD column with *n*-hexane–ethanol–TFA (86:14:0.2, vol/vol/vol). The percentage of the resultant (2'*S*)-VHA_2 relative to the total VHA (215 ± 12 pmol) is shown. The dashed line indicates the percentage of (2'*S*)-VHA_2 before the reaction.

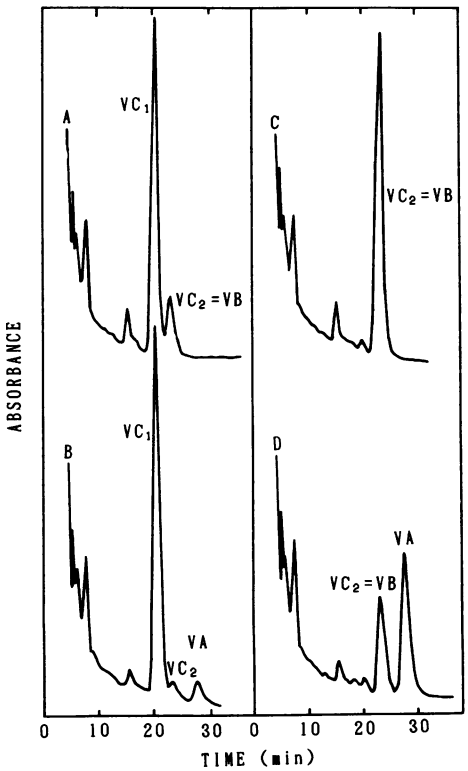

FIG. 8. Stereospecific conversion of VC_2 (=VB) to VA. The microsome fraction was incubated with VC_1 (A and B) or VC_2 (C and D) for 60 min in the absence (A and C) or presence (B and D) of NADPH and then analyzed by using a Chiralcel OJ column.

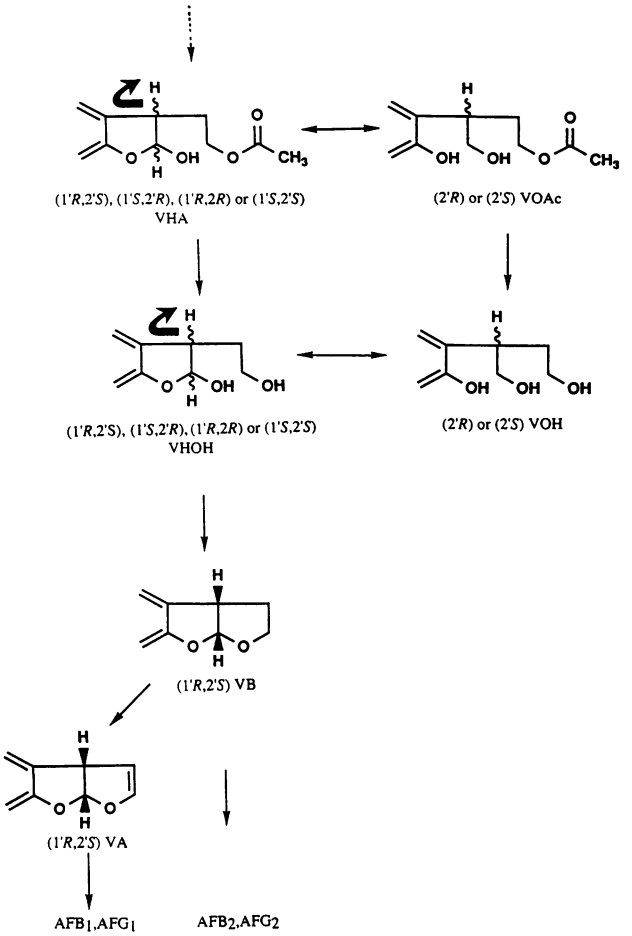

FIG. 9. Stereoconfigurational changes during the conversion of racemic VHA to (1'*R*,2'*S*)-versicolorins and finally to (1'*R*,2'*S*)-aflatoxins.

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 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). Purification of the VHA dehydrogenase activities is currently in progress.

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*)-$VHOH_1$ was nonenzymatically converted to (1'*S*,2'*R*)-(+)-VC_1, but not to (2'*S*)-VC_2 (=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 $VOAc_2$ (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 VHOH that has one aldehyde group in various polar solutions has been reported (11, 25, 26). Also, another hemiacetal substance, AFB_1 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 AFB_1 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

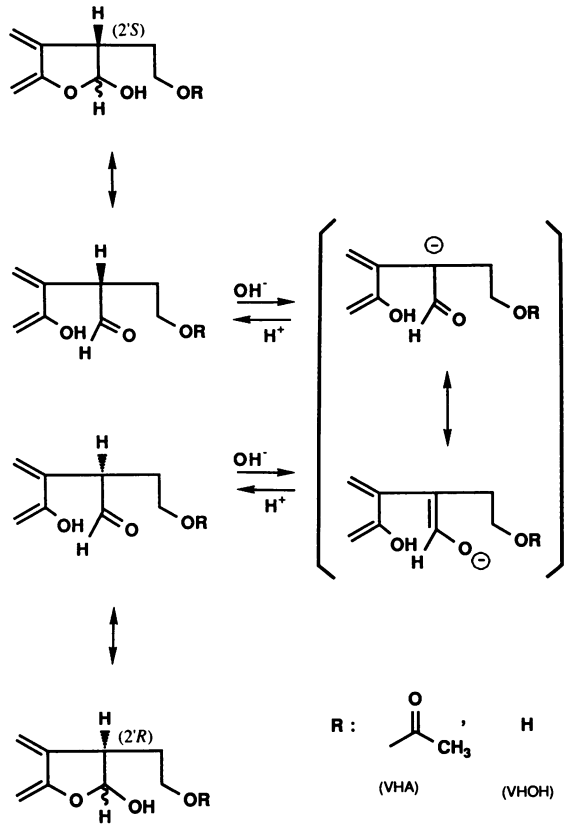

FIG. 10. Proposed racemization mechanism between (2'*S*)- and (2'*R*)-VHA or (2'*S*)- and (2'*R*)-VHOH.

acidic solution, was used. In contrast, VHA was not converted to VC by the same extraction procedure. Even when pure ethyl acetate was used to terminate the reaction, a small amount of VHOH was usually found to be converted to each corresponding enantiomer of VC (Fig. 4 and 5). Also, drying with a stream of N_2 gas easily caused cyclization from VHOH to VC (data not shown). We dried the ethyl acetate extract by storing the vessel without a lid. Even by using such procedures, we could not isolate any detectable amount of either VHOH enantiomer. These results, therefore, suggest that the general isolation of VC, instead of VB, occurs because of the artificial production of VC from VHOH that takes place during the isolation procedure from molds.

In this study, we showed that the desaturase activity also has strict stereospecificity to the (2'*S*) configuration of VB (Fig. 8). We conclude, therefore, that the (2'*S*) configuration that was selected by the cyclase reaction is further confirmed at the subsequent desaturation step. The resultant (1'*R*,2'*S*) bis-furan structure of VB or VA may be kept during the conversions following these reactions and finally may become part of aflatoxin molecules.

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