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STEREO-SELECTIVE INTERACTION OF ENANTIOMERS OF DINICONAZOLE, A FUNGICIDE, WITH PURIFIED P-450/14DM FROM YEAST

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SUMMARY: R(-) isomer of diniconazole [S-3308L, (E)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol], a newly developed fungicide strongly inhibited lanosterol 14α-demethylation catalyzed by a yeast cytochrome P-450 (P-450/14DM). On the other hand, S(+) isomer of diniconazole was a weaker inhibitor for P-450/14DM. The R(-) isomer combined with both ferric and ferrous P-450/14DM and interfered binding of CO to the cytochrome. The S(+) isomer also interacted with both forms of P-450/14DM but the absorption spectra of the S(+)-diniconazole complexes were different from those of the R(-)-diniconazole complexes. Furthermore, S(+) isomer did not significantly interfere the binding of CO to P-450/14DM. These observations suggest that P-450/14DM discriminates enantiomers of diniconazole and the R(-) isomer is more favorably fit for the active site of the cytochrome.

A yeast cytochrome P-450 (P-450/14DM) (1) which catalyzes lanosterol 14α-demethylation (2) is inhibited by many azole fungicides (3,4). Inhibition of lanosterol 14α-demethylation caused depletion of ergosterol and accumulation of lanosterol and some other 14-methylsterols, and resulted in the growth inhibition of fungal cells (3). Diniconazole [(E)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol] (S-3308L, Fig. 1), a newly developed potent azole fungicide, also inhibits ergosterol biosynthesis and accumulates 14-methylsterols in fungal cells (5). This fact suggests that diniconazole is an inhibitor for P-450/14DM. Recently, Takano et al. (6) found that the antifungal activity of the R(-) isomer of diniconazole was stronger than that of the S(+) isomer. This observation suggests a possibility that P-450/14DM interacts stereo-selectively with

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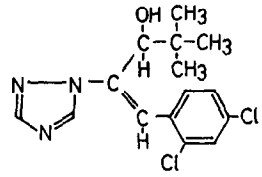

Fig. 1. Chemical structure of diniconazole (S-3308L).

R(-)-diniconazole. In order to verify this possibility, we analyzed interaction between the purified P-450/14DM of *Saccharomyces cerevisiae* and each enantiomer of diniconazole. This paper communicates lines of evidence indicating the stereo-selective interaction of R(-)-diniconazole with P-450/14DM.

EXPERIMENTAL PROCEDURES

P-450/14DM and NADPH-cytochrome P-450 reductase were purified from semi-anaerobically grown cells of *Saccharomyces cerevisiae* according to the methods of Yoshida and Aoyama (1) and Aoyama et al. (7), respectively. Lanosterol 14α-demethylase activity of the reconstituted system consisting of P-450/14DM and the P-450 reductase was assayed as described by Aoyama et al. (2). Diniconazole was added to the reaction mixture as a dimethylsulfoxide solution (5 µl) and the same volume of the solvent was added to the control. Interaction of diniconazole with P-450/14DM was analyzed spectrophotometrically. Diniconazole was added to the cytochrome solution as above. Detailed conditions of the spectrophotometric analysis are shown in the legends to figures.

RESULTS AND DISCUSSION

Both enantiomers of diniconazole inhibited lanosterol 14α-demethylase activity of the reconstituted system consisting of P-450/14DM and NADPH-cytochrome P-450 reductase. The inhibitory effect of R(-)-diniconazole was stronger than that of the S(+) isomer. R(-)-diniconazole quantitatively inhibited the activity and complete inhibition was observed when it was added at an equal concentration to P-450/14DM (Fig. 2). In contrast, S(+)-diniconazole caused about 50% inhibition at the same concentration, and 2 µM of the compound was necessary for complete inhibition (Fig. 2).

Diniconazole interacted with P-450/14DM and caused characteristic spectral changes of the cytochrome (see below). Thus, binding of diniconazole to the cytochrome could be determined spectrophotometrically. As shown in Fig. 3, both enantiomers of diniconazole interacted with ferric P-450/14DM

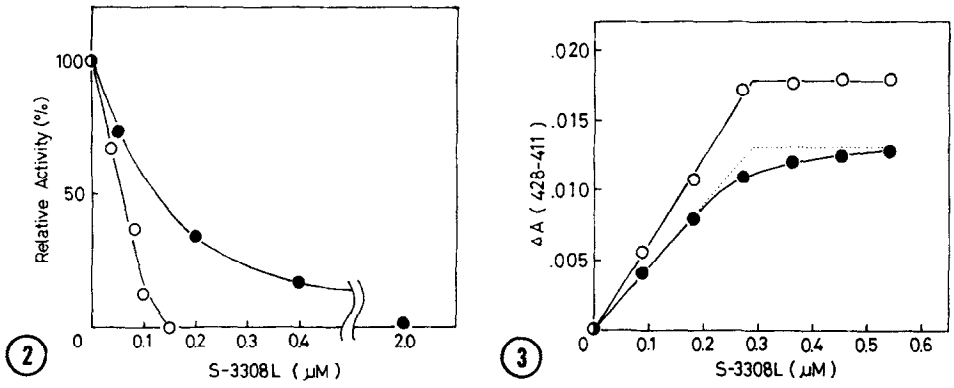

Fig. 2. Inhibition of lanosterol 14α-demethylase activity by R(-) and S(+) isomers of diniconazole (S-3308L). Lanosterol 14α-demethylase activity of a reconstituted system containing 0.14 μM of P-450/14DM was assayed as in (2) in the presence of indicated concentrations of diniconazole (S-3308L). Diniconazole was added to the reaction mixture as 5 μl of dimethylsulfoxide solution and the same volume of the solvent was added to the control. (○); R(-) isomer, and (●); S(+) isomer.

Fig. 3. Spectrophotometric titration of ferric P-450/14DM with R(-) and S(+) isomers of diniconazole (S-3308L). P-450/14DM (0.32 μM) was dissolved in 0.1 M potassium phosphate buffer, pH 7.5 containing 20% glycerol and 0.02% of Emulgen 913 and titrated with diniconazole. Formation of the diniconazole complex of P-450/14DM was detected by increasing absorbance difference between 428 and 411 nm of the resulting difference spectra. Diniconazole was added to the assay mixture as dimethylsulfoxide solution and the solvent showed no effect on the absorption spectrum of the cytochrome under the experimental conditions. (○); R(-) isomer, and (●); S(+) isomer.

with one to one stoichiometry. The difference observed between the plateau levels of these titration curves was due to the difference in the extent of red-shift and hypochromicity of the Soret band caused by each enantiomer of diniconazole (see below).

The results described above indicated that R(-)-diniconazole avidly interacted with P-450/14DM with one to one stoichiometry and inhibited lanosterol 14α-demethylation by the cytochrome. Under the experimental conditions, binding of R(-)-diniconazole to P-450/14DM was quantitative, indicating that affinity of this enantiomer to P-450/14DM was extremely high. Affinity of S(+)-diniconazole to the cytochrome was apparently weaker than the R(-) isomer though it formed one to one complex with the cytochrome. However, their *Kd* values could not be determined because concentrations of the free ligands were below the limit of detection under the experimental conditions.

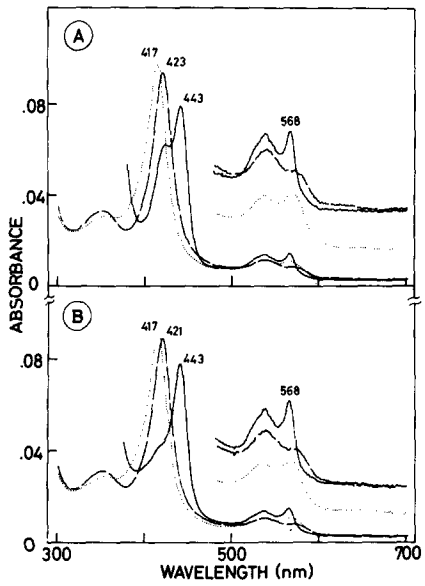

Fig. 4. Absorption spectra of diniconazole complexes of P-450/14DM. A: P-450/14DM (0.89 μM) was dissolved in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol and 0.02% Emulgen 913. R(-)-diniconazole was added at the concentration of 5 μM. B: P-450/14DM (0.82 μM) was dissolved in the same buffer as A. S(+)-diniconazole was added at the concentration of 5 μM. -------- ; free form of ferric P-450/14DM, —— —— ; diniconazole complex of the ferric form, ——— ; diniconazole complex of the ferrous form formed by reduction of the ferric complex with sodium dithionite. The spectrum was recorded 15 min after the addition of the reductant.

The spectral changes of the oxidized P-450/14DM caused by R(-)- and S(+)-diniconazole were qualitatively identical (Fig 4). Judging from the empirical rule on the relationship between ligand structure and spectral properties of cytochrome P-450 (8-11), these spectral changes indicated that both enantiomers of diniconazole interacted with the heme iron of ferric P-450/14DM by their triazole groups. However, slight but clear difference was observed on the extent of red-shift and hypochromicity of the Soret band (Fig. 4), suggesting that the mode of interaction of each enantiomer of diniconazole with the heme iron was critically different.

Upon reduction with sodium dithionite, the diniconazole complexes of ferric P-450/14DM were converted to the corresponding ferrous complexes (Fig. 4). Their absorption spectra were characterized by the unusually red-shifted Soret band at 443 nm and the sharp α-band at 568 nm. Such spectral properties were reported to be characteristic of a hexa-coordi-

nated ferrous low-spin complex of cytochrome P-450 which had a σ-donating nitrogenous ligand *trans* to the thiolate fifth ligand (12). Accordingly, each enantiomer of diniconazole must bind to the heme iron of ferrous P-450/14DM with its azole nitrogen. The absorption spectrum of the S(+)-diniconazole complex (Fig. 4B) was typical for a ferrous low-spin cytochrome P-450 which had a σ-donating nitrogenous ligand at the sixth coordination position. This fact suggested that a typical σ-bond must be formed between the coordinating nitrogen of the S(+) isomer and the heme iron. Accordingly, it could be assumed that the triazole group of the S(+) isomer coordinated almost vertically to the heme plane. The absorption spectrum of the R(-)-diniconazole complex (Fig. 4A) was somewhat atypical with a distinct shoulder at 425 nm, suggesting that the triazole ring of the R(-) isomer was not vertical to the heme plane.

The most striking difference observed between these two complexes of ferrous P-450/14DM was their reactivity with CO. Addition of CO to the S(+)-diniconazole complex readily converted the complex to the CO compound of the cytochrome, indicating that the S(+) isomer was readily replaced by CO (Fig. 5B). In contrast, the R(-)-diniconazole complex reacted slowly with CO (Fig. 5A, inset). The spectral changes shown in Fig. 5 further suggested that conversion of the R(-)-diniconazole complex to the CO compound was incomplete. These facts suggested that the R(-)-diniconazole complex of ferrous P-450/14DM was considerably stable to compare with the S(+) isomer complex. Unfortunately, ferrous P-450/14DM could not react directly with diniconazole as with other azole fungicides (4). Therefore, affinity of each enantiomer of diniconazole to ferrous P-450/14DM could not be compared.

Taken all observations together, it is concluded that P-450/14DM discriminates the configuration of C-3 of diniconazole and interacts more avidly with the R(-) isomer both in the ferric and ferrous states. Consequently, the R(-) isomer strongly inhibits lanosterol 14α-demethylase activity of the cytochrome and binding of CO to the reduced cytochrome. The

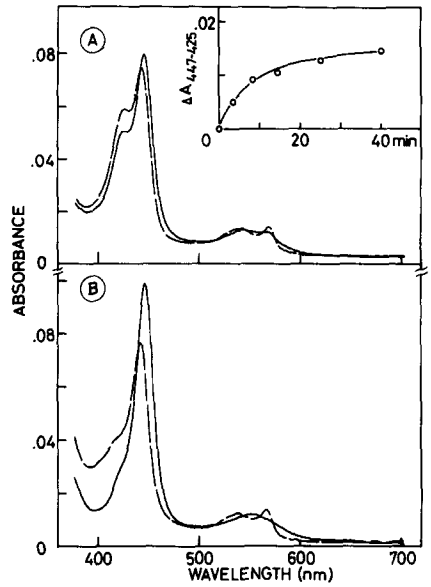

Fig. 5. Conversion of the diniconazole complexes of ferrous P-450/14DM to the ferrous CO-compound. A: The R(-)-diniconazole complex of ferrous P-450/14DM formed in the experiment of Fig. 4 was bubbled with CO for 30 sec. The resulting spectral changes were recorded successively. —— —— ; the spectrum recorded before CO bubbling, ——— ; the spectrum recorded 40 min after CO bubbling. Inset: Time course of CO binding to the complex. B: The S(+)-diniconazole complex of ferrous P-450/14DM formed in the experiment of Fig. 4 was bubbled with CO for 30 sec. —— —— ; the spectrum recorded before CO bubbling, ——— ; the spectrum recorded immediately after CO bubbling.

S(+) isomer inhibits only the catalytic activity and the inhibitory effect is lower than that of the R(-) isomer. The bulky substituent at N-1 must interact with the substrate site as in the case of other azole fungicides (3,4) while the azole group interacts with the heme iron of the cytochrome. Accordingly, the substrate site of P-450/14DM may have a conformation which fits R rather than S configuration of C-3 of diniconazole.

It was reported that ketoconazole and itraconazole inhibited both the catalytic activity and the CO binding of P-450/14DM (4,13) like R(-)-diniconazole, while triadimefon (4) and buthiobate (14) could not inhibit the CO binding as in the case of S(+)-diniconazole. Accordingly, azole fungicides may be classified into two groups based on their inhibitory effects; the one inhibits both the catalytic activity and CO binding and the other inhibits only the catalytic activity. Inhibition of lanosterol 14α-demethylation by the former is usually stronger than by the latter. Although

common structural features which determine the inhibitory effects have yet to be clarified, accumulation of the knowledge of such specific P-450/14DM inhibitors may be important to assume the structure of the active site of the cytochrome and to reveal structure-function relationship of azole fungicides.

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