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INTERACTION OF AZOLE ANTIFUNGAL AGENTS WITH CYTOCHROME P-450$_{14DM}$ PURIFIED FROM *SACCHAROMYCES CEREVISIAE* MICROSOMES

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Abstract—Mechanism of action of azole antifungal agents was studied by analyzing interaction of ketoconazole, itraconazole, triadimefon and triadimenol with a purified yeast cytochrome P-450 which catalyzes lanosterol 14α-demethylation (P-450$_{14DM}$). These antifungal agents formed low-spin complexes with P-450$_{14DM}$, indicating the interaction of their azole nitrogens with the heme iron. Affinity of these antifungal agents for the cytochrome was extremely high compared with usual nitrogenous ligands. Upon reduction with sodium dithionite, the azole complexes of ferric P-450$_{14DM}$ were converted to the corresponding ferrous derivatives. Spectral analysis of these complexes suggested that geometric orientation of the azole moiety of an antifungal agent to the ferrous heme iron was regulated by the interaction between the N-1 substituent and the heme environment. CO could not readily replace ketoconazole or itraconazole co-ordinating to the heme iron of ferrous P-450$_{14DM}$ while triadimefon and triadimenol complexes of the cytochrome were promptly converted to the CO complexes. The inhibitory effects of ketoconazole and itraconazole on the P-450$_{14DM}$-dependent lanosterol 14α-demethylation were higher than that of triadimenfon. The substituents at N-1 of the azole moieties of ketoconazole and itraconazole are extremely large while those of triadimefon and triadimenol are relatively small. Accordingly, observations described above suggest that the N-1 substituent of an azole antifungal agent regulates the mobility of the molecule in the heme crevice of ferrous P-450$_{14DM}$ and determines the inhibitory effect of the compound.

A number of azole derivatives have been developed as potent antifungal agents. Azole antifungal agents are known to inhibit ergosterol biosynthesis by yeast and fungi and to accumulate lanosterol and some other 14-methylsterols in these cells, indicating their inhibitory effect on lanosterol 14α-demethylase [1–6]. Lanosterol 14α-demethylation by yeast is a cytochrome P-450-dependent reaction [7–10] and azole antifungal agents are known to induce Type II difference spectrum on cytochrome P-450 in yeast microsomes [3, 5, 6, 11]. It was also reported that a few azole antifungal agents, such as ketoconazole and itraconazole, inhibited binding of CO to the reduced cytochrome P-450 in yeast microsomes [4, 11, 12]. These observations suggest a possibility that a cytochrome P-450 which catalyzes lanosterol 14α-demethylation is the target for azole antifungal agents. However, there is no direct evidence indicating that azole antifungal agents actually interact with the cytochrome and inhibit the activity of the cytochrome itself.

The cytochrome P-450 which catalyzes lanosterol 14α-demethylation (P-450$_{14DM}$) has been purified from *Saccharomyces cerevisiae* microsomes and characterized in detail [8, 10, 13, 14]. In this work, we examined extensively the interaction between typical azole antifungal agents and the purified P-450$_{14DM}$ to obtain further detailed information and confirmative evidence for the mode of action of azole antifungal agents. The antifungal agents used in this study were ketoconazole, itraconazole, triadimefon, and triadimenol (Fig. 1).

METHODS

P-450$_{14DM}$ (15 nmol/mg protein) and NADPH-cytochrome P-450 reductase were purified from semi-anaerobically grown cells of *S. cerevisiae* according to the methods of Yoshida and Aoyama [14] and Aoyama *et al.* [15], respectively. Ketoconazole and itraconazole were supplied by Dr H. Vanden Bossche of Janssen Pharmaceutica, Beerse. Triadimefon and triadimenol were obtained from Wako Pure Chemicals Co., Osaka. Other chemicals were of the purest reagents from commercial sources.

Interaction of azole antifungal agents with P-450$_{14DM}$ was analyzed spectrophotometrically by using a Shimadzu UV-300 recording spectrophotometer equipped with a Shimadzu SAPCOM-1 spectral-data processor. P-450$_{14DM}$ was dissolved in 0.1 M potassium phosphate buffer, pH 7.2, containing 20% glycerol. The assay mixture also contained trace Emulgen 913 which was included in the P-450$_{14DM}$ preparation [14]. Antifungal agents were dissolved in dimethylsulfoxide and added to the cytochrome solution. Further details are given in the figure legends.

Lanosterol 14α-demethylase activity of the reconstituted system consisting of P-450$_{14DM}$ and NADPH-

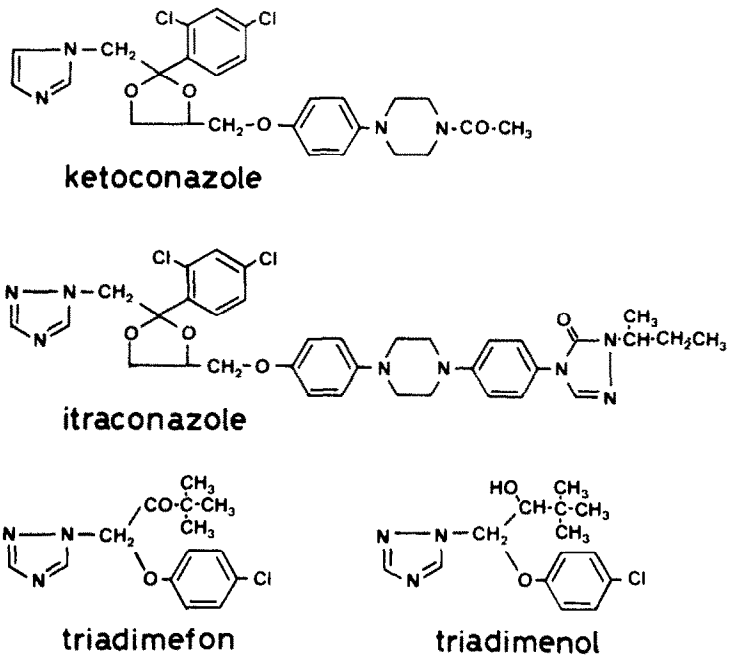

Fig. 1. Structural formula of azole antifungal agents used in this study.

cytochrome P-450 reductase was assayed by the method of Aoyama *et al.* [10].

RESULTS

Interaction of azole derivatives with ferric P-450$_{14DM}$

Ketoconazole induced a marked spectral change of ferric P-450$_{14DM}$ (Fig. 2A). This change was characterized by the red-shifts and the hypochromicity of the Soret and α bands and marked enhancement of the δ band. This type of spectral change was characteristic of binding of a basic amino-nitrogen to the sixth co-ordination position of the heme iron [16–18]. The Soret band of the ketoconazole complex was observed at 424 nm and the spectrum (spectrum B of Fig. 2A) was superimposable on that of the 1–

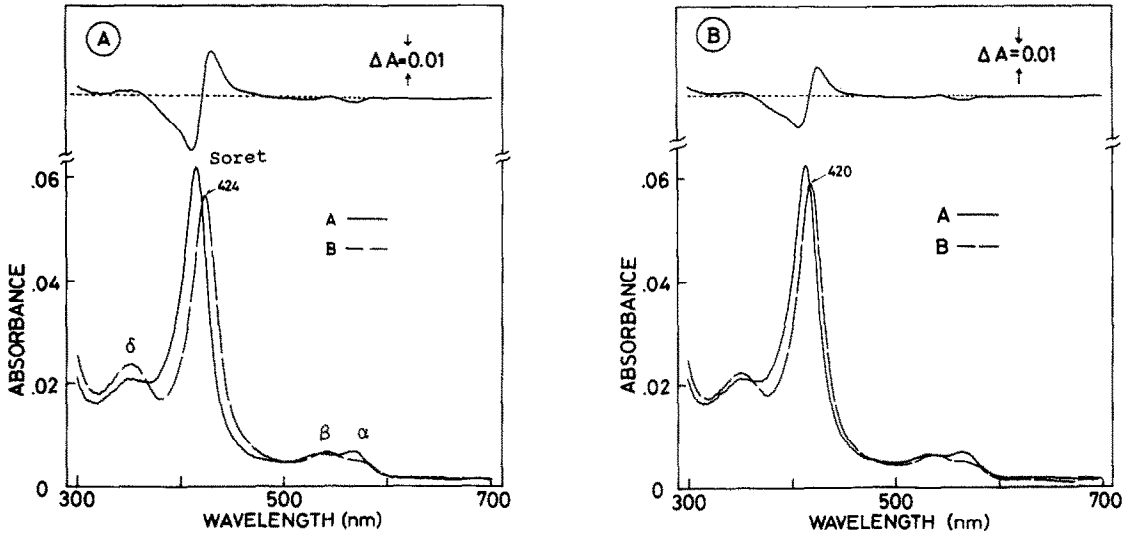

Fig. 2. Spectral change of ferric P-450$_{14DM}$ induced by ketoconazole (A) and itraconazole (B). P-450$_{14DM}$ (0.57 μM) was dissolved in 0.1 M potassium phosphate buffer, pH 7.2, containing 20% glycerol. Ketoconazole (0.7 μM) or itraconazole (0.8 μM) was added to the cytochrome as dimethylsulfoxide solution. Spectra A and B of each panel represent the free and the ketoconazole- or itraconazole-bound forms, respectively. The spectrum shown in the upper half of each panel is the difference spectrum induced by each antifungal agent.

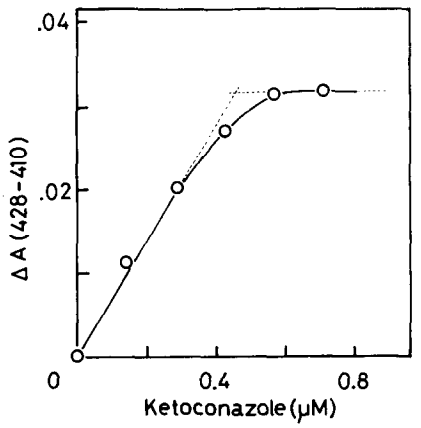

Fig. 3. Spectral titration of ferric P-450$_{14DM}$ by ketoconazole. P-450$_{14DM}$ (0.48 μM) dissolved in 0.1 M potassium phosphate buffer, pH 7.2, containing 20% glycerol was titrated with the indicated amounts of ketoconazole. Intensity of the resulting difference spectrum was plotted as a function of ketoconazole concentration.

methylimidazole complex (spectrum A of Fig. 4B). It can thus be concluded that the imidazole moiety of ketoconazole binds to the sixth co-ordination position of the heme iron and forms an artificial low-spin complex. Figure 2B represents the spectral change of P-450$_{14DM}$ induced by itraconazole. This spectral change was essentially similar to that caused by ketoconazole, indicating that the triazole moiety of itraconazole interacted with the heme iron. However, the red-shift and the hypochromicity of the Soret band were slightly but clearly smaller than those induced by ketoconazole; the Soret band of the itraconazole complex was observed at 420 nm. In relation to this the itraconazole-induced difference spectrum (the upper spectrum of Fig. 2B) was smaller than the ketoconazole-induced one (the upper spectrum of Fig. 2A). These differences must reflect the difference in the co-ordinating groups, imidazole and triazole. The spectral changes of P-450$_{14DM}$ induced by other triazole-containing antifungal agents such as triadimefon and triadimenol were superimposable on that induced by itraconazole (see spectra A of Figs 5B and C).

Figure 3 represents a typical result of the spectral titration of ferric P-450$_{14DM}$ with ketoconazole. The spectral change was linearly dependent on the amount of ketoconazole and was saturated when equimolar ketoconazole was added to P-450$_{14DM}$. This observation clearly indicated that ketoconazole and P-450$_{14DM}$ formed a one-to-one complex with very high affinity. Essentially the same results were obtained with itraconazole, triadimefon and triadimenol (data not shown). This fact indicated that these triazole antifungals also combined with the cytochrome with one-to-one stoichiometry. 1-Methylimidazole-P-450$_{14DM}$ complex showed the same spectrum as that of the ketoconazole complex

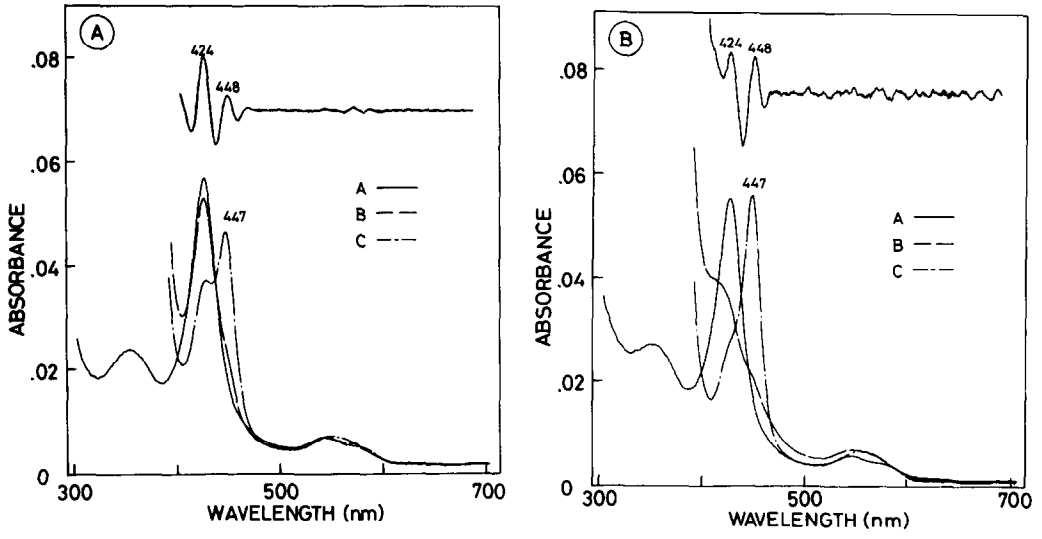

Fig. 4. Spectral changes of the ketoconazole (A) and 1-methylimidazole (B) complexes of P-450$_{14DM}$ caused by the reduction with sodium dithionite and by the addition of CO to the reduced complexes. *Panel A:* P-450$_{14DM}$ (0.57 μM) dissolved in 0.1 M potassium phosphate buffer, pH 7.2, containing 20% glycerol was converted to the ketoconazole complex with 0.7 μM ketoconazole. After recording the absorption spectrum of the ferric complex (spectrum A), a few grains of sodium dithionite was added and the absorption spectrum of the ferrous complex (spectrum B) was recorded 20 min after the addition of the reductant. CO was added to the ferrous complex and spectrum C was recorded 45 min after the addition of CO. *Panel B:* 1-Methylimidazole complex of P-450$_{14DM}$ was prepared by adding 12.5 mM of the ligand to the cytochrome solution prepared as above and the absorption spectrum of the ferric complex (spectrum A) was recorded. The complex was reduced with dithionite and the absorption spectrum of the ferrous complex (spectrum B) was recorded 20 min after the addition of the reductant. CO was added to the reduced complex and spectrum C was recorded immediately. Spectrum shown in the upper half of each panel represents the fourth derivative of spectrum B (ferrous complex).

(see spectra A of Figs 4A and B). However, the affinity of 1-methylimidazole to P-450_{14DM} was considerably lower; more than 10 mM of 1-methylimidazole was necessary to saturate the spectral change.

Interaction of azole derivatives with ferrous P-450_{14DM}

Azole antifungal agents such as ketoconazole and itraconazole could not interact with ferrous P-450_{14DM} if the cytochrome was reduced prior to the addition of the antifungal agents. However, reduced azole complexes of P-450_{14DM} could be formed by reducing the corresponding ferric complexes with sodium dithionite.

Figures 4A and B show the spectral changes of the ketoconazole and 1-methylimidazole complexes, respectively, observed upon reduction with sodium dithionite. The absorption spectra of their oxidized complexes (spectra A of Figs 4A and B) were changed to those of the corresponding reduced ones (spectra B of Figs 4A and B) within 20 min. The spectral change of the ketoconazole complex was slight and the complex seemed to remain in a partially reduced state. However, the absorption spectrum was not altered by further addition of dithionite and prolonged incubation. Consequently, Spectrum B of Fig. 4A must represent the reduced form of the ketoconazole complex. As can be seen in the fourth derivative spectra (the upper spectra of Figs 4A and B), both of these have two Soret bands at 424 and 448 nm. However, it is noteworthy that the absorption spectra (spectra B of Figs 4A and B) are considerably different from each other though ketoconazole and 1-methylimidazole co-ordinate to the heme iron with N-3 nitrogens of their imidazole moieties.

Itraconazole, triadimefon and triadimenol also interacted with ferrous P-450_{14DM} and showed the characteristic spectra shown in Fig. 5. The absorption

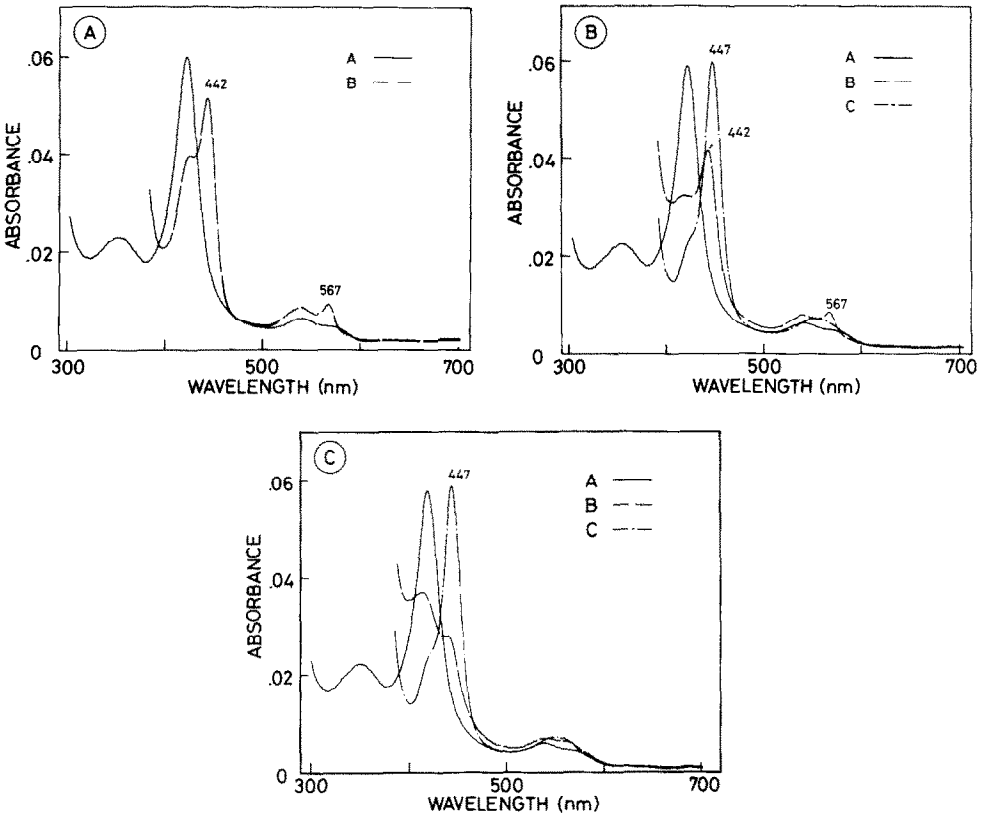

Fig. 5. Spectral changes of itraconazole (A), triadimefon (B) and triadimenol (C) complexes of P-450_{14DM} caused by the reduction with sodium dithionite and by the addition of CO to the reduced complexes. *Panel A:* P-450_{14DM} (0.57 μM) dissolved in 0.1 M potassium phosphate buffer, pH 7.2, containing 20% glycerol was converted to the itraconazole complex with 1.0 μM of itraconazole and the absorption spectrum of the ferric complex (spectrum A) was recorded. The absorption spectrum of the ferrous complex (spectrum B) was recorded 20 min after the addition of dithionite. *Panel B:* P-450_{14DM} solution prepared as above was converted to the triadimefon complex with 1.2 μM of triadimefon and the absorption spectrum of the ferric complex (spectrum A) was recorded. The absorption spectrum of the ferrous complex (spectrum B) was recorded 20 min after the addition of dithionite. CO was added to the ferrous complex and spectrum C was recorded immediately. *Panel C:* The same experiment as panel B except that triadimefon was replaced by triadimenol and the absorption spectrum of the ferrous complex (spectrum B) was recorded 50 min after the addition of dithionite.

spectra of these triazole complexes (spectra B of Figs 5A, B and C) had two Soret bands. In the itraconazole (spectrum B of Fig. 5A) and triadimefon (spectrum B of Fig. 5B) complexes, the Soret band at 442 nm was dominant and the α band was observed as a sharp, distinct peak at 567 nm. Such spectral characteristics were clearly different from those of the imidazole complexes (Figs 4A and B). However, the absorption spectrum of the triadimenol complex (spectrum B of Fig. 5C) resembled that of the 1-methylimidazole complex though triadimenol is a triazole derivative.

Inhibition of the binding of CO to ferrous P-450$_{14DM}$ by azole antifungal agents

The 1-methylimidazole (spectrum B of Fig. 4B), triadimefon (spectrum B of Fig. 5B) and triadimenol (spectrum B of Fig. 5C) complexes of ferrous P-450$_{14DM}$ were promptly and completely converted to the reduced CO complex of the cytochrome by the addition of CO as shown by appearance of the characteristic Soret peak at 447 nm (spectra C of Figs 4B, 5B and 5C). In contrast, conversion of the ketoconazole complex to the CO complex was very slow and incomplete; spectrum C of Fig. 4A was obtained at 45 min after the addition of CO and no further spectral change was observed. Furthermore, the absorption spectrum of the itraconazole complex (spectrum B of Fig. 5A) was not affected by CO. These observations indicated that ketoconazole and itraconazole interacted with ferrous P-450$_{14DM}$ so strongly as to interfere with the binding of the saturating concentration of CO to the heme iron under the experimental conditions. Unfortunately, these azoles could not react directly with ferrous P-450$_{14DM}$. Therefore, their affinity to ferrous P-450$_{14DM}$ could not be determined.

Inhibition of lanosterol 14α-demethylase activity of the reconstituted system by azole derivatives

Figure 6 shows the inhibitory effects of ketoconazole, itraconazole, and triadimefon on lanosterol 14α-demethylase activity of the reconstituted system consisting of P-450$_{14DM}$ and NADPH-cytochrome P-450 reductase purified from *S. cerevisiae* microsomes. Since these antifungal agents did not inhibit NADPH-cytochrome *c* reductase activity catalyzed by the P-450 reductase, this inhibition must be due to the direct effect of these antifungal agents on P-450$_{14DM}$ itself.

Ketoconazole and itraconazole strongly inhibited the demethylase. The inhibition was linearly dependent on their concentrations and reached 100% when they were added at a concentration equal to that of P-450$_{14DM}$. This fact indicated that these antifungal agents inhibited the demethylase activity by forming a one-to-one complex with P-450$_{14DM}$ with very high affinity. In contrast, more than 1 μM of triadimefon was required for complete inhibition, indicating its relatively low affinity for the reconstituted system.

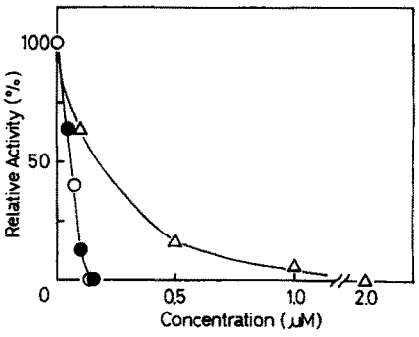

Fig. 6. Inhibition of lanosterol 14α-demethylase activity by ketoconazole, itraconazole and triadimefon. The reaction mixture containing 0.28 nmol P-450$_{14DM}$, 1.32 units NADPH-cytochrome P-450 reductase, 13 nmol lanosterol dispersed with 0.05 mg dilauroylphosphatidylcholine, 0.3 μmol NADPH, 20 μmol glucose 6-phosphate and 0.2 unit glucose-6-phosphate dehydrogenase in 2.0 ml of 0.1 M potassium phosphate buffer, pH 7.5 was incubated aerobically at 30° for 20 min in the presence of various concentrations of indicated antifungal agents. Antifungal agent was added to the reaction mixture as a dimethylsulfoxide solution (5 μl) and the same volume of the solvent was added to the control experiment. Lanosterol 14α-demethylase activity was calculated from the conversion ratio of lanosterol to 4,4-dimethylcholestatrienol determined by gas chromatographic analysis of sterols extracted from the reaction mixture (10). ○, ● and △ represent ketoconazole, itraconazole and triadimefon, respectively.

DISCUSSION

Azole antifungal agents combined with ferric P-450$_{14DM}$ with one-to-one stoichiometry. Their azole nitrogens co-ordinated to the heme iron of the cytochrome and formed low-spin azole complexes. The spectral properties of the ketoconazole (spectrum B of Fig. 2A) and the 1-methylimidazole (spectrum A of Fig. 4B) complexes were essentially identical. Similarly, no significant difference was observed among the itraconazole (spectrum B of Fig. 2B), triadimefon (spectrum A of Fig. 5B) and triadimenol (spectrum A of Fig. 5C) complexes of the cytochrome, while their spectral properties were slightly but clearly different from those of the ketoconazole and 1-methylimidazole complexes. These observations lead to the conclusion that the spectral properties of an azole antifungal agent–ferric P-450$_{14DM}$ complex is dependent on the azole species co-ordinating to the heme iron and the substituent at N-1 of the azole moiety does not affect the spectral properties of the oxidized complexes.

Affinity of azole antifungal agents for ferric P-450$_{14DM}$ was high (Fig. 3) while the affinity of 1-methylimidazole was low. Since 1-methylimidazole is the smallest imidazole derivative, the low affinity of this compound cannot be explained by steric hinderance. Therefore, some stereospecific interaction of the N-1 substituent with the apoprotein near the heme must play an important role in binding of an azole antifungal agent to ferric P-450$_{14DM}$. In addition, hydrophobic interaction between the N-1 substituent and the heme environments may also be important since azole antifungal agents are lipophilic in nature and 1-methylimidazole is a water-soluble compound.

Upon reduction with sodium dithionite, the azole complexes of ferric P-450$_{14DM}$ were converted to the

corresponding ferrous complexes (Figs 4 and 5). However, large azole antifungal agents such as ketoconazole and itraconazole could not interact directly with ferrous P-450_{14DM}. This fact seems to suggest that P-450_{14DM} closes its heme crevice on reduction and rejects large azoles approaching the heme from outside of the cytochrome.

The itraconazole and the triadimefon complexes of ferrous P-450_{14DM} are characterized by the red-shifted Soret band at 442 nm and the sharp distinct α band at 567 nm (curves B of Figs 5A and 5B). These spectral characteristics are reported to be typical for the ferrous low-spin form of P-450 having a σ-donor nitrogen ligand *trans* to the fifth thiolate ligand [19]. Since the triazole moiety is a typical ligand with σ-donor nitrogens, the above properties are quite reasonable for azole complexes of ferrous P-450_{14DM}. In contrast, the ketoconazole, triadimenol and 1-methylimidazole complexes showed neither the distinct Soret peak at 442 nm nor the sharp α peak at 567 nm though these ligands are also σ-donating in nature. The spectral properties of these complexes indicate that the modes of interaction of these ligands to the heme iron deviate from those of itraconazole and triadimefon. This deviation seems to reflect different geometric orientation of their azole moieties to the ferrous heme iron which may be determined by the interaction between their N-1 substituents and the apoprotein.

The ketoconazole and itraconazole complexes of ferrous P-450_{14DM} were not readily converted to the reduced CO complex while the triadimefon, triadimenol and 1-methylimidazole complexes were promptly converted to the CO complex. This fact indicates that the binding of ketoconazole or itraconazole to ferrous P-450_{14DM} is stronger than that of the others. As shown in Fig. 6, the inhibitory effects of ketoconazole and itraconazole on the catalytic activity of P-450_{14DM} were higher than that of triadimefon. These facts seem to suggest that the inhibitory effect of an azole antifungal agent apparently relates to its ability to interfere the binding of CO to ferrous P-450_{14DM}. Another example supporting this assumption can be found in our previous paper [20]. Buthiobate, a pyridine-containing antifungal agent, inhibited P-450_{14DM} with nearly the same potency as triadimefon, and buthiobate bound to ferrous P-450_{14DM} was readily replaced by CO.

Azole antifungal agents must interact with P-450_{14DM} both at the heme iron and at the apoprotein near the heme (Fig. 7). Since the heme crevice of ferrous P-450_{14DM} is considered to be narrower than that of the ferric form as discussed above, large antifungal agents may not move so easily in the heme crevice of the reduced cytochrome. Accordingly, geometric orientation of the azole moiety of an azole antifungal agent to the heme iron of ferrous P-450_{14DM} may be affected by the N-1 substituent and this may reflect on the spectral properties of the azole complex of ferrous P-450_{14DM} (Figs 4 and 5). The low reactivity of the ketoconazole and itraconazole complexes with CO may also be due to the low mobility of these antifungal agents in the heme crevice of the ferrous P-450_{14DM}. Thus, azole antifungal agents having large N-1 substituents such as ketoconazole and itraconazole bind firmly to the heme crevice or to the active site of P-450_{14DM} and strongly inhibit lanosterol 14α-demethylation catalyzed by the cytochrome.

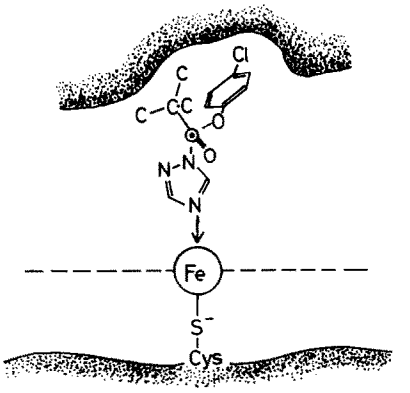

Fig. 7. A schematic representation of a possible interaction mode of an azole antifungal agent with P-450_{14DM}.

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