J. Biochem. **137**, 625–632 (2005)
DOI: 10.1093/jb/mvi073

Effects of Y132H and F145L Substitutions on the Activity, Azole Resistance and Spectral Properties of *Candida albicans* Sterol 14-Demethylase P450 (CYP51): A Live Example Showing the Selection of Altered P450 through Interaction with Environmental Compounds

Makiko Kudo[1], Miwa Ohi[1], Yuri Aoyama[3], Yuko Nitahara[3], Sung-Kee Chung[4] and Yuzo Yoshida[1,2,*]

[1]School of Pharmaceutical Sciences and [2]Institute for Bioscience, Mukogawa Women's University, Nishinomiya, Hyogo 663-8179; [3]Faculty of Engineering, Soka University, Hachioji, Tokyo 192-8577; and [4]Pohang University of Science and Technology, Pohang, 790-784, Korea

Received November 10, 2004; accepted March 6, 2005

Three variants of *Candida albicans* CYP51 (sterol 14-demethylase P450) having Y132H and/or F145L substitutions were purified and characterized to reveal the effects of these amino acid substitutions on the enzymatic properties and azole resistance of the enzyme. Y132H and F145L substitutions modified the spectral properties of the enzyme, suggesting that they caused some structural change modifying the heme environments of CYP51. Y132H and F145L substitutions increased the resistance of the enzyme to azole compounds but considerably decreased the catalytic activity. This fact represents a trade-off between acquisition of azole resistance and maintenance of high activity in the CYP51 having Y132H and F145L substitutions. A fluconazole-resistant *C. albicans* strain DUMC136 isolated from patients receiving long-term azole treatment was a homozygote of the altered *CYP51* having Y132H and F145L substitutions. However, neither of these substitutions was found in *CYP51* of wild-type *C. albicans* so far studied. These facts suggest that the azole-resistant variant having Y132H and/or F145L substitutions might be selected only under azole-rich environments because of its azole resistance and impaired catalytic activity. This may be a live example showing one of the important processes of P450 diversification, the selection of altered P450 through the interaction with environmental compounds.

Key words: azole antifungal agent, azole resistance, biodiversity, CYP51, CYP51 variant, cytochrome P450, fungus, mutation, site-directed mutagenesis, sterol 14-demethylase.

Abbreviations: CYP51, sterol 14-demethylase P450; FCZ, fluconazole; SKC-derivatives, a series of novel azole compounds synthesized by S.K. Chung and his colleagues; IC_{50}, concentration of an inhibitor necessary for 50% inhibition; SRS, substrate-recognition site.

Numerous species of P450 monooxygenase showing diversified substrate specificities exist in the biological world. These P450 monooxygenases might have been formed through gene duplications, mutations of duplicated genes and subsequent selection as a result of interaction with environmental compounds. A newly formed P450 variant might be fixed as a novel enzyme if it is advantageous to the organism. The selection of a newly formed P450 variant by environmental compounds must be a key step in P450 diversification. However, it is impossible to obtain evidence showing the existence of such a selection process from the comparative and molecular phylogenetic analysis of the existing P450s.

A live example demonstrating the formation of a functionally modified P450 through mutation and selection by environmental compounds may be obtained by examining the mutation of specific P450 species under specific conditions. Emergence of an azole-resistant variant of sterol 14-demethylase P450 (CYP51) in a pathogenic fungus under azole-rich conditions may be one of the most suitable materials for such examination. CYP51 is an essential enzyme for sterol biosynthesis that is conserved in eukaryotes (*1–4*), and fungal CYP51 is the primary target of azole antifungal agents (*5–8*). Many azole-resistant mutants of pathogenic fungi have been isolated (*9–11*), and altered *CYP51* genes with a few amino acid substitutions were found in them (*12–17*). The amino acid substitutions occurred in these CYP51s might reduce the azole susceptibility of the enzyme, and these altered CYP51s may have been selected through interaction with azole-rich environments. However, these considerations are speculative, and precise experimental evaluation of the effects of the amino acid substitutions occurring in CYP51 variants on their azole resistance, catalytic activity and molecular properties is needed.

The *CYP51* gene (called $CYP51_{DUMC}$ in this work) of a fluconazole (FCZ) resistant isolate of *Candida albicans*, strain DUMC136 (*18*), has four amino acid substitutions, D116E, K128T, Y132H and F145L, compared with the reported sequence of *C. albicans CYP51* (DDBJ/EMBL/

*To whom correspondence should be addressed at: School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien-kyuban-cho, Nishinomiya, Hyogo 663-8179. Tel: +81-798-45-9946, Fax: +81-798-41-2792 E-mail: yoshiday@mwu.mukogawa-u.ac.jp


```
                116                 128  132                 145
ATCC-2  LSDVSAEEAYKHLTTPVFGTGVIYDCPNSRLMEQKKFA
ATCC-1  *******D**********K*****************
DUMC    **********************H***********L*
MT      DD*LDQAK**-PFM**I**E**VF*ASPE*RK*M--LH
                                89          100
```

Fig. 1. **The amino acid substitutions identified in *CYP51* alleles of *C. albicans* ATCC 90028, and the *CYP51* variant of *C. albicans* DUMC136.** ATCC-1 and ATCC-2 represent the amino acid sequences encoded by the *CYP51* alleles of *C. albicans* ATCC 90028, and the former corresponds to the sequence appearing in DDBJ, EMBL and GenBank as X13296. DUMC is the amino acid sequence encoded by *CYP51* variant occurring in the FCZ-resistant *C. albicans* DUMC136. MT is the amino acid sequence of the corresponding region of *Mycobacterium tuberculosis* CYP51.

GenBank: X13296) (Fig. 1) (*14*). Two of these substitutions, D116E and K128T, were also observed in the *CYP51* alleles (called $CYP51_{ATCC\text{-}1}$ and $CYP51_{ATCC\text{-}2}$ in this work) of an azole-sensitive strain of *C. albicans*, ATCC 90028 (Fig. 1) (*14*). Comparison of these sequences suggested that $CYP51_{ATCC\text{-}1}$ and $CYP51_{DUMC}$ might be derived from $CYP51_{ATCC\text{-}2}$ by introducing two amino acid substitutions: E116D and T128K for $CYP51_{ATCC\text{-}1}$, and Y132H and F145L for $CYP51_{DUMC}$. The FCZ-resistant *C. albicans* DUMC136 is a homozygote of $CYP51_{DUMC}$, whereas *CYP51* variants having Y132H and/or F145L substitutions have not been found in azole-sensitive *C. albicans* strains so far studied (*14*). However, other amino acid substitutions including E116D and T128K were found among *CYP51* genes of these strains (*14*). These facts indicated that a CYP51 variant having either the Y132H or F145L substitution is unlikely to be selected under normal environments, but the *CYP51* variant having both of these substitutions was positively selected under FCZ-rich environments. Consequently, it is expected that detailed examination on the effects of Y132H and F145L substitutions on the enzymatic properties and azole susceptibility of CYP51 will provide useful information for considering the selection mechanism of P450 variants by environmental compounds. Therefore, we constructed two artificial *CYP51* variants having either Y132H ($CYP51_{HF}$) or F145L ($CYP51_{YL}$) substitution. These two artificial *CYP51*s, as well as $CYP51_{DUMC}$, $CYP51_{ATCC\text{-}1}$ and $CYP51_{ATCC\text{-}2}$ were expressed independently in *Escherichia coli*, and the five CYP51 variants were purified. This paper describes the results of spectrophotometric and enzymatic characterization of the purified preparations of these CYP51 variants as well as their reactivity with azole compounds. Based on these results, a possible mechanism of P450 diversification through mutation and selection by interaction with environmental compounds is discussed.

METHODS

Construction of Expression Plasmids of CYP51 Variants—The cloned $CYP51_{DUMC}$ prepared by Asai *et al.* (*14*) was used as the starting material for constructing five *CYP51* variants. Before constructing the plasmid for expressing $CYP51_{DUMC}$ in *E. coli* cells, the following modifications were made on the cloned $CYP51_{DUMC}$. The codon "CTG", which is translated as serine by *Candida* sp. (*19*, *20*) but as leucine by other organisms, was replaced with a general serine codon "TCG" by site-directed mutagenesis according to the method described by Nitahara *et al.* (*21*). One *Nde*I site (CATATG) present in the upstream fragment (see below) was converted to CTTATG by site-directed mutagenesis (*21*) to protect this site from *Nde*I digestion in the construction of the expression plasmid (see below) without amino acid substitution. The 5′-end including the initiation codon was made up to CATATG (*Nde*I site), and the following 21 nucleotides were arranged to encode the N-terminal amino acid sequence of MALLLAVF to improve the expression in *E. coli* (*22*).

The upstream fragment extending from 5′-end (*Nde*I site) to the unique *Xba*I site and including all of the above-mentioned modifications was prepared by PCR. This *Nde*I–*Xba*I fragment (upstream part) and the *Xba*I–*Hin*dIII fragment (downstream part) obtained by *Xba*I and *Hin*dIII digestion of the cloned $CYP51_{DUMC}$ were inserted together into the *Nde*I–*Hin*dIII site of pCWori$^+$ vector to construct the plasmid, pCW/DUMC, for expressing $CYP51_{DUMC}$. The expression plasmids for $CYP51_{ATCC\text{-}1}$ and $CYP51_{ATCC\text{-}2}$, pCW/ATCC-1 and pCW/ATCC-2, respectively, were constructed by replacing the *Sac*I–*Spe*I fragment of pCW/DUMC with the corresponding fragment of the cloned $CYP51_{ATCC\text{-}1}$ or $CYP51_{ATCC\text{-}2}$ (*14*, *23*). To construct two artificial *CYP51* variants having either Y132H or F145L mutation, the *Bam*HI–*Hin*dIII fragment of pCW/DUMC covering the complete coding sequence of $CYP51_{DUMC}$ was subcloned with pBluescript SK- and subjected to site-directed mutagenesis (*21*) to replace either H132 with Y to give $CYP51_{YL}$ or L145 with F to give $CYP51_{HF}$. Each of these modified fragments was inserted into the *Bam*HI–*Hin*dIII site of pCWori$^+$.

Expression of CYP51 Variants in E. coli and Preparation of the Membrane Fraction Containing the Expressed Proteins—The expression plasmids were transformed into *E. coli* JM109 and expressed as described previously (*22*, *23*). CYP51-expressing *E. coli* cells were washed with 10 mM potassium phosphate buffer (pH 7.5) containing 0.15 M NaCl and centrifuged at 15,000 × *g* for 10 min. The pellet was suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 0.5 mM EDTA and 0.25 M sucrose. The cells were disrupted by two passages of the suspension through a French press cell, and the resulting suspension was centrifuged to remove unbroken cells. The cell-free supernatant thus obtained was further centrifuged at 100,000 × *g* for 2.5 h. and the resulting pellet was suspended with 100 mM potassium phosphate buffer (pH 7.8) containing 0.5 mM EDTA and 20% glycerol to give the membrane fraction.

Purification of Expressed CYP51—The purification procedure described below could be applied to the purification of all CYP51 variants. The membrane fraction prepared as above was diluted with 100 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and 20% glycerol (buffer A) to give a protein concentration of 5.0 mg/mL. Then, 0.5 mM DTT and 1% sodium cholate were added to the suspension, and the suspension was allowed to stand for 40 min with gentle stirring at 4°C. The suspension was centrifuged at 100,000 × *g* for 2 h, and the supernatant containing solubilized CYP51 was dialyzed overnight against buffer A containing 0.5 mM

Table 1. **Summary of purification of five *C. albicans* CYP51s expressed in the membrane fraction of *E. coli*.** Five CYP51s were expressed in the membrane fraction of *E. coli* and purified as described in "METHODS."

CYP51	Specific content *nmol/mg protein* (yield %)		
	Membrane fraction of *E. coli*	EAH-Sepharose 4B eluate	DE52 eluate
$CYP51_{ATCC-1}$	0.238 (100)	8.32 (77.6)	12.9 (40.0)
$CTP51_{ATCC-2}$	0.205 (100)	8.86 (76.6)	13.2 (40.3)
$CYP51_{DUMC}$	0.186 (100)	10.1 (73.9)	12.2 (22.4)
$CYP51_{YL}$	0.0711 (100)	5.56 (68.7)	10.9 (45.6)
$CYP51_{HF}$	0.373 (100)	8.23 (58.1)	12.6 (37.0)

DTT and 0.5% sodium cholate. The dialyzed specimen was loaded onto a column of EAH-Sepharose 4B equilibrated with buffer A containing 0.5% sodium cholate, and the column was washed well with the equilibration buffer. The adsorbed CYP51 was then eluted from the column with the equilibration buffer containing 0.1% Emulgen 913. The fractions containing CYP51 were pooled and dialyzed overnight against 10 mM potassium phosphate buffer (pH 7.8) containing 20% glycerol and 0.1% Emulgen 913 (buffer B). The dialyzed solution was applied to a column of DE52 equilibrated with buffer B. CYP51 was adsorbed at the top of the column. The column was washed well with buffer B and eluted with a 0 to 150 mM concentration gradient of KCl in buffer B. The fractions containing CYP51 were collectedand dialyzed overnight against buffer B. The dialyzed eluate was applied to a second DE52 column. The CYP51 adsorbed on the column was washed with buffer B and then eluted very slowly with buffer B containing 150 mM KCl to give concentrated CYP51 preparation.

Spectrophotometric Analysis and Determination of CYP51 Content—Spectrophotometric properties of purified CYP51 were analyzed with either a Shimadzu UV-2200 or Shimadzu UV-300 recording spectrophotometer. CYP51 content was basically determined from its reduced CO-difference spectrum by using the extinction coefficient of 91.1 mM^{-1} cm^{-1} (*24*). The CYP51 concentration at each purification step was estimated by the absorbance of the Soret peak of oxidized CYP51 at 416 nm using the extinction coefficient of 110 mM^{-1} cm^{-1} (*25*). Protein was determined by the method of Lowry *et al*. (*26*) using bovine serum albumin as the standard.

Determination of the Catalytic Activity of CYP51—Lanosterol 14-demethyase activity of purified CYP51 was determined with a reconstituted system consisting of CYP51 and purified yeast NADPH-P450 reductase that was cloned and expressed in *E. coli* (Aoyama et al., unpublished). The reaction mixture contained 0.07 nmol CYP51, 1.0 unit of NADPH-P450 reductase, 23.4 nmol lanosterol dispersed with dilauroylphosphatidyl choline, and 1.0 μmol NADPH in 1.0 mL of 0.1 M potassium phosphate buffer (pH 7.2). The reaction was run aerobically at 30°C for 5 min, and stopped by saponification with methanolic KOH (*1*). Sterols extracted from the reaction mixture were analyzed by gas chromatography (*1*), and CYP51 activity was calculated from the conversion ratio of the substrate to the 14-demethylated metabolites as described previously (*1*). Azole compounds were added to the reaction mixture as 1.0 μl of dimethylsulfoxide solution. FCZ was the same preparation used in the previous work (*14*), and SKC derivatives (SKC-1, -3, -5, and -7) were prepared as described previously (*27*).

RESULTS AND DISCUSSION

Expression of Five CYP51 Genes in E. coli and Purification of the Expressed CYP51s—$CYP51_{ATCC-1}$, $CYP51_{ATCC-2}$, $CYP51_{DUMC}$, $CYP51_{YL}$ and $CYP51_{HF}$ were expressed effectively in *E. coli*, and most of the expressed CYP51s were recovered in the membrane fraction. The apparent amounts of expressed CYP51s determined spectrophotometrically ranged from 0.2 to 0.4 nmol/mg protein, except for that of $CYP51_{YL}$ (Table 1). CYP51 expressed in the membrane fraction could be solubilized by the treatment with 1.0% sodium cholate in the glycerol-containing buffer. The solubilized CYP51 was purified with the EAH-Sepharose 4B column chromatography developed for the purification of CYP51 from the microsomal fraction of *Saccharomyces cerevisiae* (*25*). However, CYP51 of *C. albicans* was unstable upon subsequent adsorption on a hydroxylapatite column, even though such a column was effective in the purification of CYP51s of *S. cerevisiae* (*25*) and rat (*22*). Therefore, the subsequent purification procedure was newly developed. The amino acid sequence predicted that *C. albicans* CYP51 would be an acidic protein, whereas CYP51s of *S. cerevisiae* and mammals are basic proteins that bind to CM-Sepharose column (*22*, *25*). Based on these characteristics, chromatography with DE52 column at pH 7.8 was employed, and the purification procedure described in Methods was established. By this procedure, the five CYP51 variants were successfully purified (Table 1). Specific contents of purified preparations except for $CYP51_{YL}$ were about 13 nmol/mg protein, and yields were over 37% except for $CYP51_{DUMC}$. All CYP51 variants showed essentially the same behavior during purification process and the final preparations were homogenous in SDS-PAGE (data not shown). These results indicated no substantial difference in stability and chromatographic behavior among the five CYP51 variants through the purification process.

Spectrophotometric Properties of CYP51 Variants—Figure 2A shows typical absorption spectra of oxidized form, reduced form and reduced CO-complex of the purified $CYP51_{ATCC-2}$, a wild-type enzyme. These spectra were essentially the same as those of CYP51 of *S. cerevisiae* (*25*), and essentially the same spectra were observed for $CYP51_{ATCC-1}$, $CYP51_{YL}$ and $CYP51_{HF}$. However, the spectral properties of $CYP51_{DUMC}$ (Fig. 2B) were slightly but clearly different from those of $CYP51_{ATCC-2}$. The Soret peak of ferric $CYP51_{DUMC}$ showed a significant red-shift to 419 nm, and the broad absorption peak around 420 nm of the ferrous form (spectrum 2 of Fig. 2B) was of weaker intensity than the corresponding peak of ferrous $CYP51_{ATCC-2}$ observed at 414 nm (spectrum 2 of Fig. 2A). These findings suggested that Y132H and F145L substi-

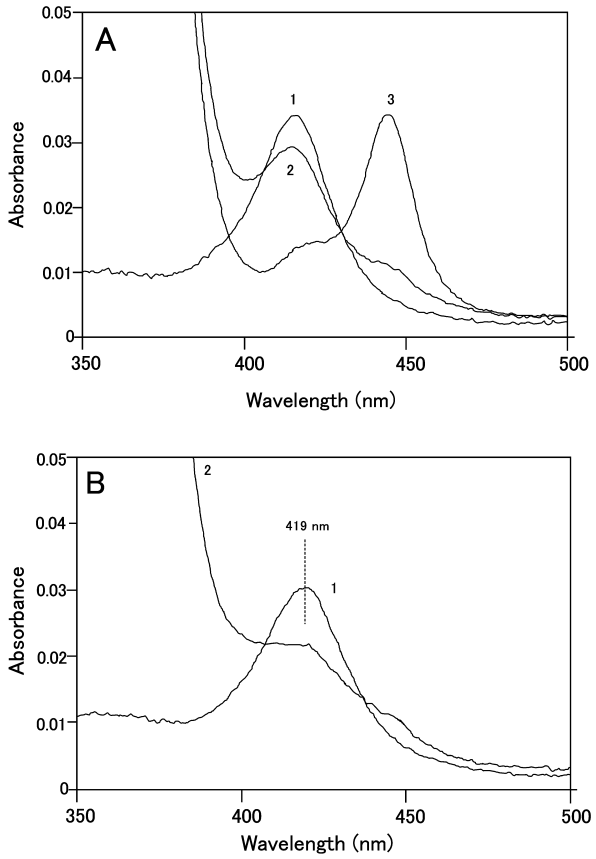

Fig. 2. **Absorption spectra of purified CYP51 preparations.** Panel A: Absorption spectra of $CYP51_{ATCC-2}$. Purified preparation of $CYP51_{ATCC-2}$ (0.31 μM) was dissolved in 10 mM potassium phosphate buffer, pH 7.8, containing 20% glycerol and 0.1% Emulgen 913. Spectra 1, 2 and 3 represent the oxidized form, the reduced form with dithionite and the reduced CO-complex, respectively. Panel B: Absorption spectra of $CYP51_{DUMC}$. Purified preparation of $CYP51_{DUMC}$ (0.28 μM) was dissolved in 10 mM potassium phosphate buffer, pH 7.8, containing 20% glycerol and 0.1% Emulgen 913. Spectra 1 and 2 represent the oxidized form and the reduced form with dithionite, respectively.

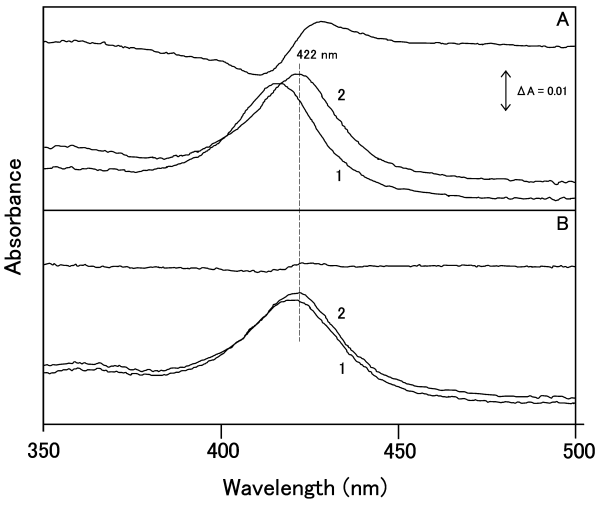

Fig. 3. **Spectral changes of $CYP51_{ATCC-2}$ and $CYP51_{DUMC}$ caused by the binding with FCZ.** Purified preparations of $CYP51_{ATCC-2}$ (0.31 μM) and $CYP51_{DUMC}$ (0.28 μM) were dissolved in 10 mM potassium phosphate buffer, pH 7.8, containing 20 % glycerol and 0.1% Emulgen 913. Panels A and B represent $CYP51_{ATCC-2}$ and $CYP51_{DUMC}$, respectively. Absorption spectrum of oxidized form (spectrum 1) was recorded. Then, 12.5 μM of FCZ was added and the absorption spectrum of its FCZ complex (spectrum 2) was recorded. Note that the absorption peak of the FCZ complex was observed at 422 nm on both $CYP51_{ATCC-2}$ and $CYP51_{DUMC}$ as indicated by the broken line. Upper part of each panel is the difference spectrum obtained by subtracting spectrum 1 from spectrum 2.

tutions significantly modified the heme environment of CYP51, causing detectable changes in the spectrophotometric properties of its ferric and ferrous forms.

Upon binding with an azole compound, the Soret peak of ferric CYP51 shows a characteristic red-shift that is caused by the coordination of azole nitrogen to the heme iron (*8*), and this spectral change is detectable as the difference spectrum called Type II. As shown in Fig. 3A, $CYP51_{ATCC-2}$ showed clear red-shift of the Soret band and a typical Type II difference spectrum upon binding with FCZ. Essentially the same FCZ-induced Type II difference spectra were observed for $CYP51_{ATCC-1}$, $CYP51_{YL}$ and $CYP51_{HF}$, but the FCZ-induced difference spectrum of $CYP51_{DUMC}$ was small, as shown in Fig. 3B. It is noteworthy that the absorption spectrum of the FCZ complex of $CYP51_{DUMC}$ (spectrum 2 of Fig. 3B) was superimposable on that of $CYP51_{ATCC-2}$ (spectrum 2 of Fig. 3A), and the low intensity of the FCZ-induced Type II difference spectrum of $CYP51_{DUMC}$ is due to the red-shifted Soret band of ferric $CYP51_{DUMC}$ (spectrum 1 of Fig. 3B). This fact indicated that the unusual spectral feature of ferric $CYP51_{DUMC}$ represented by the red-shifted Soret band (spectrum 1 of Fig. 2B) was masked by the binding of FCZ as the sixth ligand. Accordingly, the red-shifted Soret band of ferric $CYP51_{DUMC}$ might be due to modification of the interaction with the internal sixth ligand caused by Y132H and F145L substitutions. Exchange of the internal sixth ligand caused by the conformational change induced by one amino acid substitution was suggested for the similar red-shifted Soret band of a naturally occurring *S. cerevisiae* CYP51 mutant named $P450_{SG1}$ (*28*). However, the exact structural changes causing the modified spectrophotometric characteristics of $CYP51_{DUMC}$ remain unknown.

Reduction of $CYP51_{ATCC-2}$ by dithionite was slow, and the reduced CO-complex was stable under the experimental conditions (Fig. 4, bottom), and $CYP51_{ATCC-1}$, $CYP51_{YL}$ and $CYP51_{HF}$ showed similar behavior upon reduction by dithionite in the presence of CO. Since essentially the same phenomena were observed with the purified CYP51 of *S. cerevisiae* (Yoshida, Y., unpublished), slow reduction by dithionite and the stable dithionite-reduced CO-complex appear to be common features of fungal CYP51s. However, $CYP51_{DUMC}$ was rapidly reduced by dithionite, and its reduced CO-complex was readily denatured to P420 form (Fig. 4, top). These facts indicated that introduction of both Y132H and F145L substitutions considerably altered the chemical reducibility and stability of the reduced CO-complex of CYP51.

These evidence obtained by spectrophotometric examinations on CYP51 variants indicated that Y132H and F145L substitutions together caused local conforma-

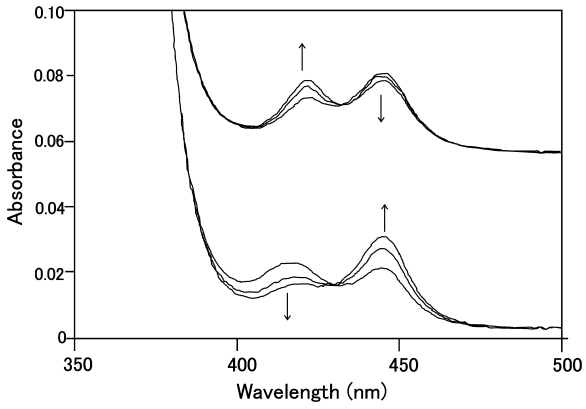

Fig. 4. **Time dependent spectral changes of $CYP51_{ATCC-2}$ and $CYP51_{DUMC}$ caused by the reduction with dithionite in the presence of CO.** Purified preparations of $CYP51_{ATCC-2}$ (0.31 μM) and $CYP51_{DUMC}$ (0.28 μM) were dissolved in 10 mM potassium phosphate buffer, pH 7.8, containing 20% glycerol and 0.1% Emulgen 913. A few grains of dithionite were added to the specimen, and CO was bubbled through the solution. The absorption spectra of $CYP51_{DUMC}$ (top) and $CYP51_{ATCC-2}$ (bottom) were recorded immediately after the CO bubbling and 5 min and 10 min later. The arrows indicate the directions of the time-dependent movements of the absorption peaks.

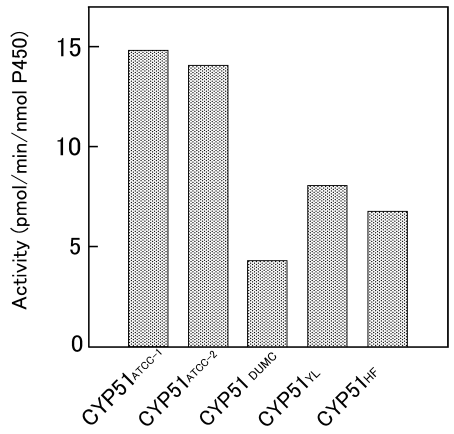

Fig. 5. **Lanosterol 14-demethylase activities of the purified preparations of five CYP51s.** Lanosterol 14-demethylase activities of the purified preparations of five CYP51s were determined as described in "METHODS."

tional change affecting the coordination structure of the heme iron, although neither substitution alone caused significant spectral change.

Catalytic Properties of the CYP51 Variants—The purified preparations of the wild-type enzymes encoded by *CYP51* alleles of *C. albicans* ATCC 90028, $CYP51_{ATCC-1}$ and $CYP51_{ATCC-2}$, showed lanosterol 14-demethylase activity with a specific activity over 10 nmol/min/mg protein upon reconstruction with yeast NADPH-cytochrome P450 reductase (Fig. 5). Introduction of Y132H or F145L substitution into $CYP51_{ATCC-2}$ considerably reduced the activity, as shown by the activities of $CYP51_{HF}$ and $CYP51_{YL}$ (Fig. 5). These amino acid substitutions seemed to have additive effects on the enzyme activity, since the activity of $CYP51_{DUMC}$ having both Y132H and F145L substitutions was less than one-third of that of $CYP51_{ATCC-2}$ (Fig. 5). As discussed in the preceding section, reduced CO-complex of $CYP51_{DUMC}$ was rapidly converted to a denatured 420 nm form. This fact suggested that the low activity of $CYP51_{DUMC}$ might be due to loss of the active enzyme by denaturation. However, this possibility was unlikely, since the time course of the $CYP51_{DUMC}$-mediated reaction was linear up to 10 min of incubation. Therefore, the local conformational change induced by Y132H and F145L substitution caused unfavorable effects on the catalytic activity of the enzyme.

Azole Susceptibility of the CYP51 Variants—Susceptibility to FCZ of the wild-type and three variants of CYP51 is summarized in Fig. 6. The wild-type $CYP51_{ATCC-2}$ was highly sensitive to FCZ with apparent IC_{50} of less than 0.03 μM, whereas $CYP51_{DUMC}$ showed FCZ resistance with apparent IC_{50} of about 0.3 μM. These results were essentially the same as those described in the preliminary communication (*23*). Interestingly, the Y132H and F145L variants had different FCZ susceptibilities. $CYP51_{YL}$ showed high FCZ resistance comparable to that of $CYP51_{DUMC}$, whereas $CYP51_{HF}$ showed low FCZ resistance (Fig. 6). These findings suggested that the FCZ resistance of $CYP51_{DUMC}$ was dominantly dependent on F145L substitution, while Y132H substitution might contribute little to the FCZ resistance of $CYP51_{DUMC}$. Essentially the same result was reported by Bellamine *et al.* (*29*). Since Y132H substitution further reduced the activity of CYP51 decreased by F145L substitution (Fig. 5), the question arose why $CYP51_{DUMC}$ having Y132H substitution was selected. Moreover, Y132H is an amino acid substitution frequently found in naturally occurring CYP51 variants of azole-resistant *C. albicans* mutants (*16*).

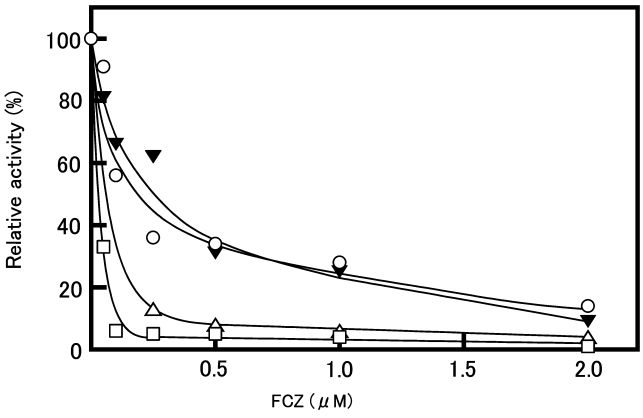

Fig. 6. **Inhibitory effect of fluconazole on the lanosterol 14-demethylase activity of the purified preparations of CYP51.** Lanosterol 14-demethylase activity of purified CYP51 was determined as described in "METHODS." FCZ was added to the reaction mixture as 1 μl of DMSO solution, and the same volume of the solvent was added to the control. Open squares, $CYP51_{ATCC-2}$; open triangles, $CYP51_{HF}$; solid inverted triangles, $CYP51_{YL}$; and open circles, $CYP51_{DUMC}$.

To find a clue to solve the above question , the effects of a series of novel azole compounds (*27*), here called SKC derivatives, on the CYP51 variants were examined. Inhibitory effects of four compounds (SKC-1, -3, -5, and -7, Fig. 8) on CYP51 are summarized in Fig. 7. It is clear from these results that $CYP51_{ATCC-2}$ was most sensitive and $CYP51_{DUMC}$ was most resistant to all SKC deriva-

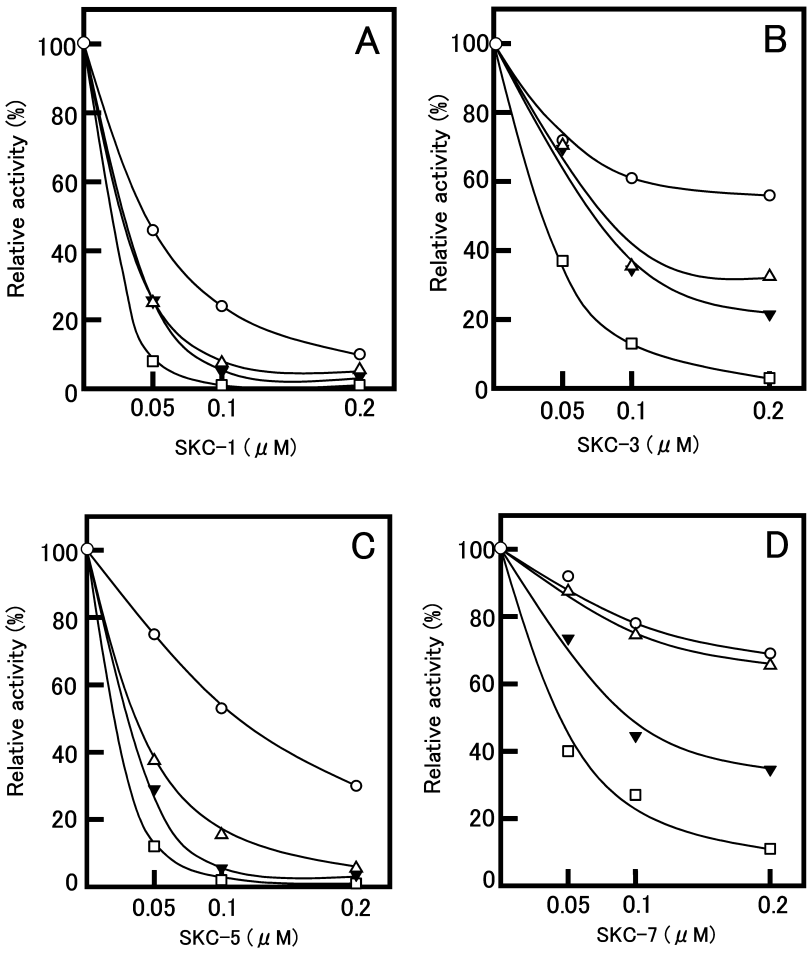

Fig. 7. **Inhibitory effect of SKC derivatives on the lanosterol 14-demethylase activity of the purified preparations of CYP51.** Lanosterol 14-demethylase activity of purified CYP51 was determined as described in Methods. SKC derivatives were added to the reaction mixture as 1 μL of DMSO solution, and the same volume of the solvent was added to the control. Panels A, B, C, and D represent inhibitory effects of SKC-1, 3, 5, and 7, respectively. Open squares, $CYP51_{ATCC\text{-}2}$; open triangles, $CYP51_{HF}$; solid inverted triangles, $CYP51_{YL}$; and open circles, $CYP51_{DUMC}$.

tives. This indicated that Y132H and F145L substitutions occurring in $CYP51_{DUMC}$ enhanced its resistance not only to FCZ but also to SKC derivatives. However, the inhibitory effects of the SKC derivatives on $CYP51_{HF}$ and $CYP51_{YL}$ depended the derivative. $CYP51_{HF}$ showed high resistance to SKC-7, whereas $CYP51_{YL}$ was sensitive to this compound (panel D of Fig. 7). This fact suggested that the Y132H substitution plays the dominant role in the high SKC-7 resistance of $CYP51_{DUMC}$, although this substitution was not necessary for the FCZ resistance of $CYP51_{DUMC}$ (Fig. 6). $CYP51_{HF}$ also showed higher resistance than $CYP51_{YL}$ to inhibition by SKC-5 (panel C of Fig. 7). However, the SKC-5 resistance of $CYP51_{HF}$ was markedly lower than that of $CYP51_{DUMC}$, suggesting that both Y132H and F145L substitutions synergistically enhanced the SKC-5 resistance of CYP51. In contrast, both $CYP51_{HF}$ and $CYP51_{YL}$ showed similar moderate resistance to SKC-1 and 3, and resistance of $CYP51_{DUMC}$ to these compounds seemed to be the sum of them (panel A and B of Fig. 7).

These observations together indicate that the effect of Y132H or F145L substitution on azole resistance of CYP51 depends on the structure of the azole compound (Fig. 8), but that introduction of both of these substitutions induces high resistance of the enzyme to multiple azole compounds.

General Considerations—Amino acid sequence alignment of $CYP51_{ATCC\text{-}2}$ and CYP51 of *M. tuberculosis* (*30*) showed that Y132 and F145 of the former correspond respectively to F89 and L100 of the latter (see Fig. 1). X-ray crystallographic analysis of the FCZ complex of *M. tuberculosis* CYP51 (*31*) showed that F89 and L100 were included in the BC loop and had no direct interaction with the bound FCZ. Since the fundamental architecture of *M. tuberculosis* and *C. albicans* CYP51s may be conserved, Y132 and F145 of $CYP51_{ATCC\text{-}2}$ are considered to be included in the BC loop and have no direct interaction with the azole molecule in the active site. Podust *et al.* (*31*) described that the BC loop is one of the most dynamic regions affecting the conformation of *M. tuberculosis* CYP51 molecule. Accordingly, Y132H and F145L substitutions occurring in BC loop must affect the conformation of *C. albicans* CYP51, and this possibility is supported by the unusual spectrophotometric properties of $CYP51_{DUMC}$ (Figs. 2 through 4). Thus, it can be inferred that the conformational modification induced by Y132H and F145L substitutions must be the reason for the high azole resistance of $CYP51_{DUMC}$ (Figs. 6 and 7). However, the same conformational modification might cause considerable impairment of the catalytic activity (Fig. 5). Therefore, the acquisition of azole resistance is a trade-off against maintenance of high catalytic activity for $CYP51_{DUMC}$. Podust *et al.* (*32*) inferred that the mutations of fungal *CYP51* inducing azole resistance occurred to preserve the catalytic activity. Their inference was simply deduced from the fact that the mutation hot-spots of

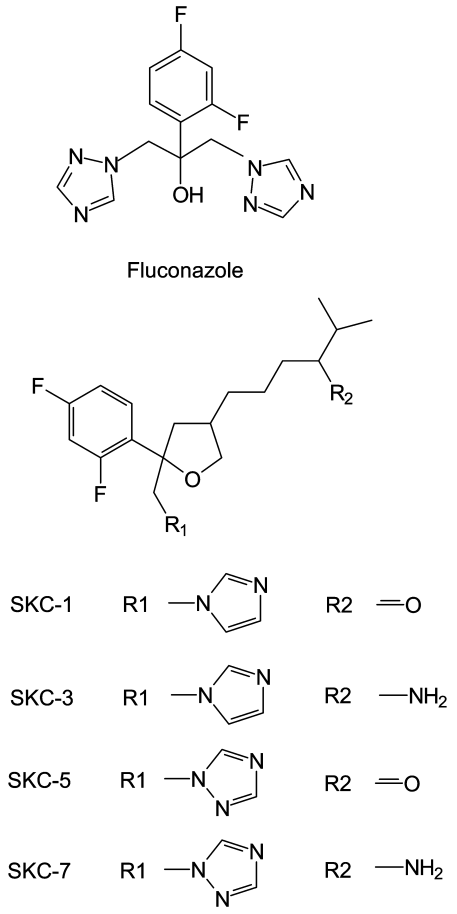

Fig. 8. **Structural formulas of the azole compounds used in this study.**

*CYP51*s found in the azole resistant isolates (*16*) existed outside of the putative SRSs. The present discussion based on extensive examination of CYP51 variants having Y132H and/or F145L substitutions suggests that the inference of Podust *et al*. (*32*) may not always be adequate to illustrate the general mechanism for the emergence of azole-resistant CYP51 variants in pathogenic fungi. As described by Asai *et al*. (*14*), azole-resistant *C. albicans* DUMC136 was a homozygote of $CYP51_{DUMC}$, suggesting that this CYP51 variant was positively selected in azole-rich environments such as patients receiving long-term azole treatment. However, neither Y132H nor F145L substitution necessary for azole resistance of $CYP51_{DUMC}$ has been found in the *CYP51* alleles occurring in azole sensitive natural isolates of *C. albicans* so far studied (*14*). Results obtained in this work revealed that the molecular and catalytic properties of $CYP51_{ATCC-1}$ and $CYP51_{ATCC-2}$ encoded by *CYP51* alleles of *C. albicans* ATCC 90028 were essentially the same in spite of the existence of two amino acid substitutions at the116th and 128th positions (Fig. 1). Both $CYP51_{ATCC-1}$ and $CYP51_{DUMC}$ were derived from $CYP51_{ATCC-2}$ by mutations introducing two amino acid substitutions (Fig. 1), and each of these mutations might occur randomly with similar possibility. Consequently, the following scenario can be written for the emergence of azole-resistant CYP51. *CYP51* of *C. albicans* underwent frequent mutations, but functionally disadvantageous mutations were eliminated to maintain the essential function of CYP51 in eukaryotic life, and only neutral mutations were allowed to form allelic polymorphisms. However, in azole-rich environments such as in the azole-treated patients, azole resistance might be the dominant factor for selecting *CYP51* variants, and an altered *CYP51* encoding an azole-resistant form such as $CYP51_{DUMC}$ was selected, even if this involved disadvantageous changes to its enzymatic properties. This is a live example demonstrating the occurrence of dynamic alterations of P450 and the selection of resulting P450 variants through interaction with environmental compounds. Although the present example is the formation of an altered enzyme showing resistance to the inhibitors, essentially the same process may occur in the formation of a new enzyme showing novel or expanded substrate specificity, which is the core process of P450 diversification.

The authors thank to Mr. K. Asai and Dr. K. Okonogi of Takeda Chemicals, Co. Ltd. for their kind permission to use cloned $CYP51_{DUMC}$ and FCZ preparation in this work.

REFERENCES

1. Aoyama, Y., Yoshida, Y., and Sato, R. (1984) Yeast cytochrome P-450 catalyzing lanosterol 14α-demethylation, II. Lanosterol metabolism by purified $P450_{14DM}$ and by intact microsomes. *J. Biol. Chem.* **259**, 1661–1666
2. Yoshida, Y. (1993) Lanosterol 14α-demethylase (cytochrome $P450_{14DM}$) in *Cytochrome P450* (Schenkman, J.B. and Greim, H., eds.) pp. 627–639, Springer-Verlag, New York
3. Trzaskos, J.M., Bowen, W.D., Shafiee, A., Fischer, R.T., and Gaylor, J.L. (1984) Cytochrome P-450-dependent oxidation of lanosterol in cholesterol biosynthesis. Microsomal electron transport and C-32 demethylation. *J. Biol. Chem.* **259**, 13402–13412
4. Taton, M. and Rahier, A. (1991) Properties and structural requirements for substrate specificity of cytochrome P-450-dependent obtusifoliol 14α-demethylase from maize (Zea mays) seedlings. *Biochem. J.* **277**, 483–492
5. Vanden Bossche, H. (1985) Biochemical targets for antifungal azole derivatives: Hypothesis on the mode of action in Current Topics in *Medical Mycology* (McGinnis, M.R., ed.) Vol. **1**, pp.313–351, Springer-Verlag, New York
6. Yoshida, Y. and Aoyama, Y. (1987) Interaction of azole antifungal agents with cytochrome $P450_{14DM}$ purified from *Saccharomyces cerevisiae* microsomes. *Biochem. Pharmacol.* **36**, 229–235
7. Vanden Bossche, H., Marichal, P., Gorrens, J., Geerts, H., and Janssen, P.A. (1988) Mode of action studies. Basis for the search of new antifungal drugs in antifungal drugs. *Ann. NY Acad. Sci.* **544**, 191–207
8. Yoshida, Y. (1998) Current topics in *Medical Mycology* Vol. 2 (McGinnis MR ed.) pp. 388–418, Springer-Verlag, New York
9. Sanglard, D., Kuchler, K., Ischer, F., Pagani, J.L., Monod, M., and Bille, J. (1995) Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob. Agents Chemother.* **39**, 2378–2386
10. Johnson, E.M., Warnock, D.W., Luker, J., Porter, S.R., and Scully, C. (1995) Emergence of azole drug resistance in *Candida* species from HIV-infected patients receiving prolonged fluconazole therapy for oral candidosis. *J. Antimicrob. Chemother.* **35**, 103–114
11. Rex, J.H., Rinaldi, M.G., and Pfaller, M.A. (1995) Resistance of *Candida* species to fluconazole. *Antimicrob. Agents Chemother.* **39**, 1–8

12. Lamb, D.C., Kelly, D.E., Schunck, W.H., Shyadehi, A.Z., Akhtar, M., Lowe, D.J., Baldwin, B.C., and Kelly, S.L. (1997) The mutation T315A in *Candida albicans* sterol 14α-demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. *J. Biol. Chem.* **272**, 5682–5688
13. Lamb, D.C., Kelly, D.E., White, T.C., and Kelly, S.L. (2000) The R467K amino acid substitution in *Candida albicans* sterol 14α-demethylase causes drug resistance through reduced affinity. *Antimicrob. Agents Chemother.* **44**, 63–67
14. Asai, K., Tsuchimori, N., Okonogi, K., Perfect, J.R., Gotoh, O., and Yoshida, Y. (1999). Formation of Azole-resistant *Candida albicans* by Mutation of Sterol 14-demethylase P450. *Antimicrob. Agents Chemother.* **43**, 1163–1169
15. Loffler, J., Kelly, S.L., Hebart, H., Schumacher, U., Lass-Florl, C., and Einsele, H. (1997) Molecular analysis of cyp51 from fluconazole-resistant *Candida albicans* strains. *FEMS Microbiol. Lett.* **151**, 263–268
16. Sanglard, D., Ischer, F., Koymans, L., and Bille, J. (1998) Amino acid substitutions in the cytochrome P-450 lanosterol 14α-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob. Agents Chemother.* **42**, 241–253
17. White, T.C. (1997) The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14α-demethylase in *Candida albicans*. *Antimicrob. Agents Chemother.* **41**, 1488–1494
18. National Committee for Clinical Laboratory Standards. (1992) Reference method for broth dilution antifungal susceptibility testing of years. Proposed standard document M27-P. National Committee for Clinical Laboratory Standards, Villanova, Pa.
19. Sugiyama, H., Ohkuma, M., Masuda, Y., Park, S.M., Ohta, A., and Takagi, M. (1995) In vivo evidence for non-universal usage of the codon CUG in *Candida maltosa*. *Yeast* **11**, 43–52
20. White, T.C., Andrews, L.E., Maltby, D., and Agabian, N. (1995) The ″universal″ leucine codon CTG in the secreted aspartyl proteinase 1 (SAP1) gene of *Candida albicans* encodes a serine in vivo. *J. Bacteriol.* **177**, 2953–2955
21. Nitahara, Y., Kishimoto, K., Yabusaki, Y., Gotoh, O., Yoshida, Y., Horiuchi, T., and Aoyama, Y. (2001) The amino acid residues affecting the activity and azole susceptibility of rat. CYP51 (sterol 14-demethylase P450). *J. Biochem.* **129**, 761–768
22. Nitahara, Y., Aoyama Y., Horiuchi T., Noshiro M., and Yoshida Y. (1999) Purification and characterization of rat Sterol 14-demethylase P450 (CYP51) expressed in *Escherichia coli*. *J. Biochem.* **126**, 927–933
23. Aoyama, Y., Kudo, M., Asai, K., Okonogi, K., Horiuchi, T., Gotoh, O., and Yoshida, Y. (2000) Emergence of fluconazole-resistant sterol 14-demethylase P450 (CYP51) in *Candida albicans* is a model demonstrating the diversification mechanism of P450. *Arch. Biochem. Biophys.* **379**, 170–171
24. Omura, T. and Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**, 2370–2378
25. Yoshida, Y. and Aoyama, Y. (1984) Yeast cytochrome P-450 catalyzing lanosterol 14α-demethylation. I. Purification and spectral properties. *J. Biol. Chem.* **259**, 1655–1660
26. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
27. Chung, S.K., Lee, K.W., Kang, H.I., Yamashita, C., Kudo, M., and Yoshida, Y. (2000) Design and synthesis of potential inhibitors of the ergosterol biosynthesis as antifungal agents. *Bioorg. Med. Chem.* **8**, 2475–2486
28. Ishida, N., Aoyama, Y., Hatanaka, R., Oyama, Y., Imajo, S., Ishiguro, M., Oshima, T., Nakazato, H., Noguchi, T., Maitra, U.S., Mohan, V.P., Sprinson, D.B., and Yoshida, Y. (1988) A single amino acid substitution converts cytochrome $P450_{14DM}$ to an inactive form, cytochrome $P\text{-}450_{SG1}$: Complete primary structures deduced from cloned DNAs. *Biochem. Biophys. Res. Commun.* **155**, 317–323
29. Bellamine, A., Lepesheva, G.I., and Waterman, M.R. (2004) Fluconazole binding and sterol demethylation in three CYP51 isoforms indicate differences in active site topology. *J. Lipid Res.* **45**, 2000–2007
30. Aoyama, Y., Horiuchi, T., Gotoh, O., Noshiro, M., and Yoshida, Y. (1998) CYP51-like gene of *Mycobacterium tuberculosis* actually encodes a P450 similar to eukaryotic CYP51. *J. Biochem.* **124**, 694–696
31. Podust, L.M., Poulos, T.L., and Waterman, M.R. (2001) Crystal structure of cytochrome P450 14α-sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. *Proc. Natl. Acad. Sci. USA* **98**, 3068–3073
32. Podust, L.M., Stojan, J., Poulos, T.L., and Waterman, M.R. (2001) Substrate recognition sites in 14α-sterol demethylase from comparative analysis of amino acid sequences and X-ray structure of *Mycobacterium tuberculosis* CYP51. *J. Inorg. Biochem.* **87**, 227–235