Structural Diversities of Active Site in Clinical Azole-bound Forms between Sterol 14α-Demethylases (CYP51s) from Human and Mycobacterium tuberculosis*

Koji Matsuura**, Shiro Yoshioka††, Takehiko Toshia**, Hiroshi Hori††, Koichiro Ishimori‡‡, Teizo Kitagawa**, Isao Morishima‡, Norio Kagawa††, and Michael R. Waterman‡

From the **Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto 615-8510, Japan, ††Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, ‡‡Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki 444-8787, Japan, and †††Graduate School of Engineering Science, Osaka University, Toyonaka 560-8531, Japan

Received for publication, November 18, 2004, and in revised form, December 14, 2004

Published: JBC Papers in Press, December 20, 2004, DOI 10.1074/jbc.M413042200

To gain insights into the molecular basis of the design for the selective azole anti-fungals, we compared the binding properties ofazole-based inhibitors for cytochrome P450 sterol 14α-demethylase (CYP51) from human (HuCYP51) and Mycobacterium tuberculosis (MtCYP51). Spectroscopic titration of azoles to the CYP51s revealed that HuCYP51 has higher affinity for ketoconazole (KET), an azole derivative that has long lipophilic groups, than MtCYP51, but the affinity for fluconazole (FLU), which is a member of the anti-fungal armamentarium, was lower in HuCYP51. The affinity for 4-phenylimidazole (4-PhIm) to MtCYP51 was quite low compared with that to HuCYP51. In the resonance Raman spectra for HuCYP51, the FLU binding induced only minor spectral changes, whereas the prominent high frequency shift of the bending mode of the heme vinyl group was detected in the KET- or 4-PhIm-bound forms. On the other hand, the bending mode of the heme propionate group for the FLU-bound form of MtCYP51 was shifted to high frequency as found for the KET-bound form, but that for 4-PhIm was shifted to low frequency. The EPR spectra for 4-PhIm-bound MtCYP51 and FLU-bound HuCYP51 gave multiple g values, showing heterogeneous binding of the azoles, whereas the single g values were observed for other azole-bound forms. Together with the alignment of the amino acid sequence, these spectroscopic differences suggest that the region between the B’ and C helices, particularly the hydrophobicity of the C helix, in CYP51s plays primary roles in determining strength of interactions with azoles; this differentiates the binding specificity of azoles to CYP51s.
Structural Diversities between Azole-bound Forms of CYP51

CYP51-active sites based on primary sequence analysis and available structures for bacterial P450s (20, 21). In addition, until recently known forms of mammalian and plant CYP51s were membrane-bound microsomal enzymes (22), which complicated structural studies of this protein by x-ray crystallography. Recently, a soluble CYP51 ortholog was found in MtCYP51 and the molecular structures of the 4-phenylimidazole (4-PhIm)-bound and FLU-bound forms of MtCYP51 are resolved (23), revealing that the bent I helix and the region between the B' and C helices interact with 4-PhIm and FLU. However, the structure of MtCYP51 has so far been the only available structure in CYP51s, and structural information of other CYP51s are still limited. For discovery of novel anti-fungal or anti-bacterial azoles that do not inhibit human enzymes, the detailed characterization of the structural diversities in the heme pockets between mammalian and bacterial CYP51s is essential.

To obtain insight into molecular mechanisms for specificity of azoles, we focused on two CYP51s from different species and characterized their azole binding properties by spectroscopies including absorption, resonance Raman, and EPR spectra. One of the CYP51s we focused on is MtCYP51, whose three-dimensional structure with and without azole inhibitors is available (23, 24). Another CYP51 we examined here is derived from human (HuCYP51). Although HuCYP51 plays key roles in metabolizing steroids (25) and is one of the human CYPs that is not desired to be inhibited (25), the enzyme is a microsomal membrane-bound protein, and the difficulty in crystallization prevents us from elucidating the secondary and tertiary structures. The structural and functional comparison between the two CYP51s would provide some new insights into design of novel azoles with high specificity to bacterial and fungal CYPs.

To investigate the inhibitory effects of the azoles to the two CYP51s, we measured dissociation constants \( K_d \) of three heme iron-ligated azole inhibitors, FLU, KET, and 4-PhIm, from the absorption titration. In addition, we also estimated \( K_d \) of 2-phenylimidazole (2-PhIm), an inhibitor supposed to bind to CYP51 without the ligation to the heme iron (26). The heme environmental structures of azole-bound and unbound CYP51s were characterized by the EPR and resonance Raman spectroscopies, which provide information on the heme environmental changes induced by the azole binding. Based on the functional and structural comparison of two CYP51s, the structural diversities in the heme pockets between mammalian and bacterial CYP51 are discussed.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—MtCYP51 was expressed in DH5α and purified as described previously (23, 27–30). We purified the enzyme by histidine-tagged column (Amersham Biosciences) with 50 mM phosphate buffer (pH 7.4) containing 20% glycerol. Expression of HuCYP51 was performed in DH5α as previously reported (28).

To avoid the oligomerization, the membrane-spanning leader sequence was deleted from the N terminus in HuCYP51. Furthermore, we introduced the L247Q (Leu-247→Glu) mutation into the N terminus-deleted HuCYP51, which enabled us to purify the enzyme without detergents (31). In this study, HuCYP51 represents the L247Q mutant of the truncated form, not the wild-type enzyme. The activity of this truncated mutant (HuCYP51) is the same as full-length wild type human CYP51\(^2\) as reported in other microsomal P450s (32, 33), and the mutation would not induce significant structural change on the heme environmental structure of the human enzyme.

Supernatants of lysate from the cells expressing CYP51s was diluted with 50 mM Tris-Cl (pH 7.4) containing 20% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 20 μM mercuric thiolate and the mixture was applied onto the Ni\(^{2\+}\)-nitroacetic acid-agarose (Qiagen) column equilibrated with the same buffer. The protein was eluted with 50 mM Tris-Cl (pH 7.4) containing 80 mM imidazole and applied onto a hydroxyapatite Bio-Gel (Bio-Rad) column equilibrated with the same buffer. After being washed with equilibrating buffer, HuCYP51 was eluted with a linear gradient of 10–250 mM phosphate buffer (pH 7.4) containing 20% glycerol. UV-Visible spectra indicated that the histidine tag is not coordinated to the heme iron in MtCYP51 and HuCYP51 (23, 27–30).

**Spectroscopic Measurements**—Electric absorption spectra of the enzymes were recorded on a Lambda 19 spectrometer (PerkinElmer Life Sciences). To determine the \( K_d \) values for the azole binding, we subtracted the spectrum of the inhibitor-bound form from that of the inhibitor-free form (27, 28). Dilution effects of solvents of azoles were accounted for by multiplication of the spectra by the appropriate factor. The concentration of azoles and the peak absorbance differences were then fitted to the following Equation 1,

\[
\frac{1}{A} = \left( \frac{K_d}{A_{max}} \right) [I] + \frac{1}{A_{max}}
\]

where \([I]\) is the concentration of azole and \(A_{max}\) and \(A_{max}\) are the observed difference in absorption at given concentrations of azole ligand and the difference in absorption at saturation, respectively.

For the measurements of the resonance Raman spectra, the azole-bound and -unbound ferric forms were excited at 413.1 nm with a krypton ion laser (BeamLok 2060, Spectra Physics) at room temperature (34). The Raman scattering was detected with a single polychromator (DG-1000, Riteau) equipped with a liquid nitrogen-cooled charge-coupled device (CCD3200, Astromed, or CCD-100PB, Princeton Instruments). The spectra were analyzed by the same method as reported previously (34). The enzyme concentration for the resonance Raman measurements was 0.05 mM. The concentrations of the inhibitors we used for the measurements were 5, 2.5, 2.5, and 5 mM for 4-PhIm, FLU, KET, and 2-PhIm, respectively (33).

EPR spectra were measured on a Varian E-12 spectrometer equipped with an Oxford ESR-900 liquid helium cryostat. The microwave frequency was X-band (9.22 GHz), and the measurements were carried out at 15 K. The microwave power and modulation were 10 mW and 1 mT, respectively, at 15 K. The concentration of the enzymes was 0.1 mM. The concentrations of inhibitors for the EPR measurements were 3, 3, 3, and 10 mM for 4-PhIm, FLU, KET, and 2-PhIm, respectively.

**RESULTS**

**Azole Titration by Absorption Spectra**—To elucidate specificity of the azole inhibitors, we determined the dissociation constant of the azoles by using spectral titration in the Soret region. The Soret peaks of the azole-bound ferric forms were detected at 417 nm in both of MtCYP51 and HuCYP51. The peak at 417 nm is characteristic of the 6-coordinate low spin state (6cLS) (27, 28), indicating that a water molecule is coordinated to the heme iron. By the addition of 4-PhIm, KET, and FLU, the Soret peaks were red shifted to 422, 425, and 420 nm, respectively (data not shown). Such a red shift in the Soret peak was also found for the binding of imidazole to P450cam (35), and these red shifts (3–8 nm) are derived from the ligation of the aromatic nitrogen of the azoles to the heme iron (27–29).

The \( K_d \) values were calculated by fitting the double reciprocal plot from the azole-induced absorbance changes (27–30).

\(^2\)G. Lepesheva, unpublished results.
Calculated $K_a$ values were summarized in Table I. The dissociation constants of MtCYP51 were virtually identical to those in previous study (5 $\mu$M for KET and 10 $\mu$M for FLU) (27, 28). Both of the enzymes showed the highest and lowest affinities for KET (19 $\mu$M for MtCYP51 and 8.0 $\mu$M for HuCYP51) and 2-PhIm (2100 $\mu$M for MtCYP51 and 9100 $\mu$M for HuCYP51), respectively. The extremely low affinity for 2-PhIm can be ascribed to no ligation to the heme iron (26). The high affinity for KET and FLU has been supposed to be caused by the interactions between side chains of amino acid residues in the inhibitor binding site and the lipophilic groups of FLU and KET (27–30). Although the azole compounds having lipophilic groups have high affinity, the specificity of the azole compounds is significantly different between the two enzymes. Whereas 4-PhIm and KET exhibit higher affinity for HuCYP51 than for MtCYP51, FLU can bind more tightly to MtCYP51. This indicates that the inhibitor binding sites would be significantly different in these two CYP51 enzymes.

Such different environments of the inhibitor binding sites between two enzymes are also evident by the spectral changes in the titration of 2-PhIm. Based on Equation 1, the plots of absorbance changes at 396 and 417 nm against the azole concentrations showed the 1:1 binding of 2-PhIm to CYP51s (data not shown), but 2-PhIm cannot coordinate to the heme iron because of the structural constraint (26). In MtCYP51, the addition of 2-PhIm increased the absorbance at 396 nm in compensation for the decrease at 417 nm as illustrated in Fig. 2A. This indicates the structural transition from the 6cLS state to the 5-coordinate high spin state (5cHS). The intensity of the Soret peaks at 396 and 417 nm was almost the same in the saturated condition, showing that the fraction of the 5cHS state was ~50%. Such a transition of the spin state was also detected in the binding of isoniazid and benzimidazole to CYP51s (9), and these azole compounds are supposed to kick out the water molecule coordinating to the heme iron without the ligation of these azoles. Thus, addition of 2-PhIm displaces the coordinated water molecule from the heme iron in MtCYP51, resulting in the formation of the 5cHS state. On the other hand, addition of 2-PhIm to HuCYP51 induced only minor spectral changes in the Soret region (Fig. 2B). In 2-PhIm-bound HuCYP51, the water molecule is still ligated to the heme iron to keep the 6cLS state. The difference of the amino acid residues in the inhibitor binding sites between the two enzymes alters the binding position of 2-PhIm, leading to different spin states in the presence of 2-PhIm.

The Electronic Structure of Heme in the Azole-bound Forms, Resonance Raman Spectra—To follow the heme environmental changes associated with the azole binding, we measured resonance Raman spectra of the ferric enzymes with and without the azoles. Fig. 3 shows the high frequency region of the inhibitor-unbound and -bound ferric state with 413.1 nm excitation in which the heme oxidation state marker ($v_6$), spin state marker ($v_2$), and the lines susceptible to the spin state and coordination structure ($v_{10}$ and $v_{10a}$) were detected (36–42). In the inhibitor-unbound ferric state of MtCYP51 (Fig. 3A, a), $v_2$, $v_6$, and $v_{10}$ appeared at 1583, 1504, and 1638 cm$^{-1}$, respectively, corresponding to the 6cLS state (37, 41). The same spectral pattern was observed for HuCYP51. These observed frequencies of the marker lines in the two CYP51s were almost identical to those in CYP101, CYP102, and CYP121, which indicates that the heme of CYP51s are planar as found for other CYP enzymes (37–39).

By binding of 4-PhIm, FLU, or KET, the $v_6$ lines for both of the enzymes were slightly but definitely shifted to the lower wave numbers (from 1504–1501 cm$^{-1}$ for MtCYP51 and from 1502–1500 cm$^{-1}$ for HuCYP51), whereas other marker lines such as $v_2$ and $v_{10}$ were not perturbed. Previous studies revealed that the $v_6$ line is quite sensitive to the donor atom of the axial ligand (40, 41). Although the $v_6$ line is also supposed to reflect the donor atom of the heme iron (39), the $v_6$ line in the CYP51s was less sensitive compared with the $v_6$ line. In thromboxane synthase (CYP5), the binding of imidazole shifted the $v_6$ line from 1503–1502 cm$^{-1}$, and a similar shift in the $v_6$ line was also observed for imidazole-ligated P450csm (from 1503–1500 cm$^{-1}$) (40, 41). The azole-induced low frequency shift of the $v_6$ line in the CYP51s therefore indicates the binding of the nitrogen atom of these azole compounds to the heme iron. In contrast, addition of 2-PhIm induced no significant shift in the $v_6$ line for HuCYP51, implying that 2-PhIm cannot be ligated to the heme iron. In 2-PhIm-bound MtCYP51 however, an additional $v_6$ line appeared at 1488 cm$^{-1}$, which is characteristic of the 5cHS state (37, 38). Appearance of the 5cHS state was also indicated in the absorption spectra (Fig. 2A).

In addition to these spectra for the high frequency region, the resonance Raman spectra in the low frequency region demonstrate structural perturbations on heme peripheral groups by the azoles binding (43, 44) (Fig. 4). Several lines including stretching modes of the porphyrin skeleton and bending modes of heme peripheral groups are detected in the low frequency region (36, 43, 44). The most prominent change by the addition of the azoles was found in the bending mode of the vinyl groups ($\delta_{\text{vinyl}}$). In MtCYP51 (Fig. 4A), the $\delta_{\text{vinyl}}$ lines appeared at 413 cm$^{-1}$ for the azole unbound form, whereas the 4-PhIm-, FLU-, or KET-bound forms set the $\delta_{\text{vinyl}}$ line at 426 cm$^{-1}$. Because the Raman shift of the $\delta_{\text{vinyl}}$ line is sensitive to orientation of the heme vinyl groups, the wave number shift in the $\delta_{\text{vinyl}}$ line suggests orientational perturbation of the vinyl groups upon the binding of the azoles. The binding of 4-PhIm, FLU, or KET perturbs the environmental structure of the heme vinyl group in MtCYP51. Although a shoulder peak appeared around 426 cm$^{-1}$ by addition of 2-PhIm, the major $\delta_{\text{vinyl}}$ line was still detected at 413 cm$^{-1}$, indicating that the environmental changes around the vinyl groups by binding of 2-PhIm are rather small compared with those by other azoles. The two $v_6$ lines appeared at 1488 and 1503 cm$^{-1}$ in 2-PhIm-bound MtCYP51, although this did not reflect the behavior of $\delta_{\text{vinyl}}$ line.

Another Raman line sensitive to the azole binding is the bending mode of the propionate groups ($\delta_{\text{prop}}$). The Raman shift of the $\delta_{\text{prop}}$ line has been reported to reflect formation of a hydrogen bond between the heme propionate group and an amino acid residue in the heme pocket (36, 40, 45). The azole-unbound form of MtCYP51 exhibited the $\delta_{\text{prop}}$ line at 386 cm$^{-1}$. Slight shifts to 388 and 389 cm$^{-1}$ were observed for the FLU- and KET-bound MtCYP51, whereas the $\delta_{\text{prop}}$ line for the 4-PhIm- and 2-PhIm-bound forms appeared at 384 and 379 cm$^{-1}$, respectively. Although such shifts in the $\delta_{\text{prop}}$ line might be caused by the cleavage of the hydrogen bond of the heme propionate group with surrounding amino acid residues, the typical shift associated with the cleavage of the hydrogen bond is the low frequency shift of ~10 cm$^{-1}$ as found for thromboxane synthase and myoglobin (40, 45). Therefore for MtCYP51 it is more likely that the hydrogen bond with the heme propionate group is not disrupted by the binding of the azoles. Presumably the binding of the azoles to MtCYP51 induced some structural changes near the propionate groups of the porphyrin ring, but

| TABLE I Calculated $K_a$ values ($\mu$M) of azole compounds in MtCYP51 and HuCYP51 |
|-----------------|-----|-----|-----|-----|
|                 | MtCYP51 | FLU | 4-PhIm | 2-PhIm |
| KET             | 19 ± 1.9 | 20 ± 2 | 290 ± 29 | 2100 ± 210 |
| HuCYP51         | 8.0 ± 0.8 | 120 ± 12 | 190 ± 19 | 9100 ± 910 |
the structural perturbation is rather small and depends on the molecular structure of the azoles.

In HuCYP51, the two bending modes, δ_vinyl and δ_prop, are also susceptible to be influenced by the azole binding. However, the spectral perturbations by the azole binding in HuCYP51 are substantially different from those in MtCYP51 as displayed in Fig. 4B. Although the binding of 4-PhIm or KET shifted the δ_vinyl line from 420 to 432 cm⁻¹, the binding of FLU did not affect the position of the δ_vinyl line. The shifts of the δ_prop line by the azole bindings in HuCYP51 are also quite different from those in MtCYP51. 4-PhIm, FLU, and KET showed the high frequency shifted δ_prop line in HuCYP51. These spectral changes in the two enzymes suggest that the interactions of the azoles with the vinyl and propionate groups are different in the two CYP51s.

It should be noted here that the spectral patterns of the FLU- and KET-bound forms of MtCYP51 are quite similar, whereas the close spectral similarity was found for the 4-PhIm- and KET-bound forms of HuCYP51. Considering that 4-PhIm is a poor inhibitor for MtCYP51 and that the binding of FLU to HuCYP51 was also rather weak compared with that of another lipophilicazole, KET, the spectral pattern in the low frequency region would be related with the binding affinity of the azole inhibitors. The positions of the lines in MtCYP51 and HuCYP51 were summarized in Tables II and III, respectively.

The Electronic States of Heme Iron in Azole-bound Forms, EPR Spectra—To further characterize the azole binding in the two CYP51s and to elucidate the molecular mechanism for the specific azole binding, the EPR spectra for the azole-unbound and -bound forms of the CYP51s were examined. Fig. 5 shows the EPR spectra of the azole unbound, 4-PhIm-, FLU-, and KET-bound forms of MtCYP51 (Fig. 5A) and HuCYP51 (Fig. 5B) at 15 K. The sets of the g values (g_x, g_y, g_z) of azole-unbound MtCYP51 (A, a) and HuCYP51 (B, a) were 2.42, 2.26, 1.91, and 2.40, 2.25, 1.92, respectively, which are slightly different from those of other CYP51s such as CaCYP51 (2.43, 2.25, 1.90) (13). The slight but definite difference in the g values of these two CYP51s reflect the different environment of the vinyl groups because the δ_vinyl line in the resonance Raman spectra shows the significant frequency shift between the azole-unbound MtCYP51 and HuCYP51. As reported previously (13), the heme environmental structure of CYP51s is almost retained in broad
range of the species corresponding to the 6cLS state, but the electronic state of the heme iron depends on the interactions of the heme peripheral groups and the surrounding amino acid residues.

By binding of the azole inhibitors to the heme iron, \( g_x \) and \( g_z \) values were shifted to high and low field, respectively, for both of the enzymes. These shifts can be interpreted as decreased heme rhombicity because of the coordination of the nitrogen atom of the azole ring (35, 46–48). The decreased rhombicity was also reported in the 4-PhIm-bound form of P450cam (2.45/2.5, 2.25, 1.89) (46). On the other hand, the addition of 2-PhIm did not induce significant changes in the low spin region of the EPR spectra (data not shown), supporting no ligation of 2-PhIm to the heme iron. The only spectral change by the addition of 2-PhIm was the appearance of a small signal at 7.96 in MtCYP51, which is derived from the \( S/H11005 \) species (35, 46–48) and is consistent with the results of the absorption and resonance Raman spectra.

Although the \( g \) values of the KET-bound form were almost identical in the two enzymes, the spectral pattern was substantially different in the 4-PhIm- and FLU-bound forms. The binding of 4-PhIm induced two sets of EPR signals, 2.55, 2.26, 1.86 and 2.47, 2.26, 1.90 in MtCYP51 (Fig. 5A, b). Such discrete sets of the \( g \) values were also observed for the 4-PhIm bound form of P450cam (46) and the imidazole bound form of endothelial nitric-oxide synthase (47, 48), which are ascribed to two orientations of the imidazole ring relative to the heme plane. The two sets of the \( g \) values therefore correspond to multiple binding orientations of the azole inhibitor and suggest that the binding specificity of 4-PhIm is lower than that of other azoles in MtCYP51. In the FLU-bound form of MtCYP51, the \( g \) values were not shifted from those of the azole unbound form, but the significant signal broadening was observed. The binding of FLU perturbs the electronic state of the heme iron in MtCYP51.

It is quite interesting that the multiple sets of the \( g \) values were also found for the azole-bound HuCYP51, but the azole inhibitor showing the multiple sets of the \( g \) values is FLU, not
4-PhIm. In addition to the two sets of the $g$ values ($g = (2.53, 2.26, 1.88), (2.46, 2.26, 1.89)$), some other unresolved EPR signals around $g = 2.4$ and 1.9 were also detected in the FLU-bound HuCYP51. These multiple sets of the $g$ values in the FLU-bound form of HuCYP51 imply that the binding specificity of FLU in HuCYP51 is lower than that in MtCYP51.

**DISCUSSION**

**Correlation of the Inhibitor Binding Modes and Spectroscopic Properties of Two CYP51s**—As clearly shown by resonance Raman spectroscopy, the binding of 4-PhIm to MtCYP51 is different from that of FLU and KET in that the low frequency shifted $\delta_{\text{prop}}$ line was observed, although these three azoles can coordinate to the heme iron. The low frequency shifted $\delta_{\text{prop}}$ line was also encountered in the 2-PhIm-bound form in which 2-PhIm is not coordinated to the heme iron. The ligation of 4-PhIm to the heme iron in MtCYP51 would therefore be rather weak, corresponding to the large $K_d$ value in the titration experiment. The low affinity of 4-PhIm to the heme iron of MtCYP51 is also supported by the two sets of the EPR signals in the 4-PhIm-bound form. As reported previously (47, 48), multiple sets of the $g$ values in the EPR spectrum for the inhibitor-bound forms imply heterogeneous binding of the inhibitor, clearly showing the weak and less specific binding of 4-PhIm to MtCYP51.

On the other hand, it is FLU, not 4-PhIm, that shows the unique spectroscopic property in HuCYP51. The resonance Raman spectrum of FLU-bound HuCYP51 was quite similar to that of the azole unbound form. The bending mode of the heme vinyl group, $\delta_{\text{vinyl}}$, was not shifted by binding of FLU, whereas clear high frequency shifts were detected in the 4-PhIm- and KET-bound forms. The EPR spectrum for FLU-bound HuCYP51 gave multiple sets of the $g$ values as found for 4-PhIm-bound MtCYP51, indicative of the weak and less specific inhibitor binding of FLU to HuCYP51. In fact, the $K_d$ value of FLU in HuCYP51 is extremely large ($120 \mu M$) compared with another lipophilic azole, KET ($8.0 \mu M$) (Table I). However, the affinity of 4-PhIm ($190 \mu M$) was lower than that of FLU in HuCYP51, whereas 4-PhIm-bound HuCYP51 showed a high frequency shift in the $\delta_{\text{vinyl}}$ line and a single set of the $g$ values. The lipophilic group of FLU would increase the affinity to HuCYP51 as found for MtCYP51, but some specific interactions between FLU and surrounding amino acid residues would be lost in HuCYP51, leading to less specific binding. Thus, the spectroscopic property and binding mode of the azoles are well correlated, suggesting that the structural factors affecting the resonance Raman and EPR spectra also regulate the binding mode of the azoles for the CYP51 enzymes.

**Structural Factors Affecting the Bending Modes of Vinyl and Propionate Groups in Resonance Raman Spectra**—As discussed above, the structural perturbations around heme vinyl and propionate groups in resonance Raman spectra are well correlated, suggesting that the structural factors affecting the resonance Raman and EPR spectra also regulate the binding mode of the azoles for the CYP51 enzymes.
propionate groups affect the affinity of the azoles for the CYP51 enzymes. Based on the crystallographic structure of MtCYP51 (23), possible amino acid residues interacting with 2-vinyl and 4-vinyl groups are Phe-387 and Phe-399, respectively (Fig. 6). By binding of the azoles to MtCYP51, the positional shift was observed for Phe-399, perturbing the interaction with the 4-vinyl group (23). Although Phe-399 is located on the L helix and far from the inhibitor binding site, a hydrogen bond between Gln-403 located near Phe-399 on the L helix and Ser-261 on the I helix constituting the inhibitor binding site is formed (Fig. 6a). It is therefore plausible that the azole binding induces some structural changes of the I helix and the structural perturbation on the I helix is propagated to the L helix through the hydrogen bond between the two helices, leading to the positional changes of Phe-399 and the shift of the $\delta_{\text{prop}}$ line in the resonance Raman spectra of the azole-bound CYP51s.

Another sensitive marker for the azole binding is the $\delta_{\text{prop}}$ line. As shown in the x-ray structure of MtCYP51, a highly conserved hydrogen bond network including Gln-72 (near the B' helix), Tyr-76 (near the B' helix), Arg-326 (1–4 sheet), His-392 (near the L helix), and heme propionate groups is formed in the AB' loop and the L helix (Fig. 6b) (23). These hydrogen bonds are supposed to be essential to maintain the integrity of the inhibitor binding site (23). The C helix is also located near the heme propionate group and sensitive to the inhibitor binding (23) (Fig. 6c). Because of the large thermal fluctuation, side chains of amino acid residues on the C helix would not interact with the heme peripheral group in the azole-unbound form, whereas the temperature factors for the side chain of some amino acid residues on the C helix were decreased to form hydrogen bonds of the heme 6-propionate group with Lys-97 and Arg-95 on the C helix in the 4-PhIm- and FLU-bound forms of MtCYP51, respectively (23). It is therefore plausible that the binding of the inhibitor induces structural perturbation in the region of the AB' loop and reduces the structural fluctuation of the C helix, which alters the hydrogen bond network including the heme propionate groups and results in the frequency shifts of the bending mode of the heme propionate group.

**Structural Origin for the Specificity of Azoles**—As discussed in the previous section, the azoles interact with the side chains of the amino acid residues on the I helix and in the region between the B' and C helices. It is likely that the different inhibitor specificity between two CYP51s would be based on the amino acid sequence of these regions. One clue to identify the key amino acid residues for inhibitor specificity is the binding property of FLU. The amino acid sequence of the I helix is rather conserved and the sequence homology between MtCYP51 and HuCYP51 is high, but Phe-255 in MtCYP51 is replaced with Leu-310 in HuCYP51 (Fig. 7a). The replacement of a bulky phenylalanine residue with a flexible leucine residue would enlarge the inhibitor binding site, which relieves the steric repulsion to the inhibitors and results in the enhanced affinity of HuCYP51 for 4-PhIm and KET. However, affinity of FLU was drastically decreased in HuCYP51, which cannot be explained by the reduced steric hindrance in HuCYP51. Although we cannot exclude the possibility that some favorable interactions between Phe-255 and FLU such as $\pi-\pi$ stacking of the aromatic rings are formed only in MtCYP51, the crystal structure of FLU-bound MtCYP51 shows that the phenyl ring of Phe-255 is not parallel to 2,4-difluorophenyl or the azole ring of FLU (23), and it is unlikely that specific interactions are disrupted by the substitution of the amino acid residue in HuCYP51.

An alternative interaction site with azoles is the region between the B' and C helices. The crystal structure of the MtCYP51-FLU complex indicates that the 2,4-difluorophenyl...
Structural Diversities between Azole-bound Forms of CYP51

ring of FLU is located near Arg-96 and Leu-100 in the inhibitor binding site (Fig. 7b). The structure of the 4-PhIm-bound form also revealed that Tyr-76, Phe-78, Met-79, and Phe-89 form a hydrophobic binding site for the phenyl group of 4-PhIm. Although the amino acid residues of the hydrogen bond network constituting the inhibitor binding site, which includes the propionate group, Tyr-76, Arg-326, and His-392, in MtCYP51 are completely conserved in CYP51s, the sequence homology for the C helix is relatively low, and the hydrophilic patch including Arg-95, Arg-96, and Lys-97 in MtCYP51 is not conserved (Fig. 7a).

In HuCYP51, these hydrophilic amino acid residues are replaced with hydrophobic amino acid residues (Val-151, Phe-152, and Leu-153).

The amino acid substitution of the C helix in HuCYP51 therefore enhances hydrophobicity for the inhibitor binding site, leading to the high affinity of the hydrophobic group in 4-PhIm and KET for HuCYP51. However, FLU has the hydrophilic triazole rings and hydroxyl group, which would reduce the affinity for the hydrophobic inhibitor binding site. In addition, hydrophilic interactions are also suggested between the fluorine atom of difluorophenyl group in FLU and Arg-96 because the carbon-fluorine bond is significantly polarized (49).

The negatively charged character of the fluorine atom might be caused by the carbon-fluorine bond is significantly polarized (49). Furthermore, amino acid substitution of the B helix can also be a factor to regulate the inhibitor specificity of CYP51s. Although most of the amino acid residues are conserved in the B helix, amino acid residues interacting with the azoles in MtCYP51, Phe-78 and Met-79, are changed to Leu and Thr in HuCYP51, respectively (Fig. 7a). These amino acid residues form a pocket for the phenyl group of 4-PhIm, and the amino acid replacement of Phe-78 and Met-79 with Leu and Thr, respectively, would enlarge the pocket and stabilize the binding of 4-PhIm in HuCYP51.

Thus, we can conclude that the region between the B' and C helices is the key region for the azole recognition in MtCYP51 and HuCYP51. This region is one of the hot spots for mutation in CYP51s, and some of the mutants are azole resistant (6, 18), and HuCYP51. This region is one of the hot spots for mutation in CYP51s, the region between the B’ and C helices were exchanged in the two CYP51s is further required to confirm the suggestion. The detailed characterization of the interactions of the azoles with the region between the B’ and C helices of CYP51s in the target bacteria and host would pave the way for new azole inhibitors with high specificity and reduced side effects.

Acknowledgments—We thank Dr. Eric Johnson, Scripps Research Institute, for advice leading to the HuCYP51 mutation L247Q, which leads to purification of the truncated form without the use of detergents. The L247Q mutant expression plasmid was constructed by Drs. Brian Laden and Li Lei. We thank Dr. Takeshi Uchida for the assistance of the resonance Raman measurements and acknowledge Dr. Satoshi Takahashi for advice on the manuscript. S. Y. thanks all of the members of the Waterman laboratory for kind advice and fruitful discussions.

REFERENCES

1. Yoshida, Y., Aoyama, Y., Noshiero, M., and Osamu, G. (2000) Biochem. Biophys. Res. Commun. 273, 799–804
2. Lamb, D. C., Kelly, D. E., and Kelly, S. L. (1998) FEBS Lett. 425, 263–265
3. Koster, R. T., Stans, S. H., Johnson, P. R., Ko, S. S., Magolda, R. L., Gaylor, J. L., and Trzaskos, J. M. (1998) J. Lipid Res. 39, 1621–1632
4. Fischer, R. T., Trzaskos, J. M., Magolda, R. L., Ko, S. S., Brox, C. S., and Larsen, B. (1991) J. Biol. Chem. 266, 6114–6128
5. Trzaskos, J. M., Brown, V. O., Salles, A., Fischer, R. T., and Gaylor, J. L. (1984) J. Biol. Chem. 259, 13402–13412
6. Lamb, D. C., Kelly, D. E., and Kelly, S. L. (1999) Drug Resist. Updat. 2, 190–202
7. Debejal, N., Fink, M., and Rozman, D. (2003) Arch. Biochem. Biophys. 409, 159–171
8. Souter, A., McLeen, K. J., Smith, W. E., and Munro, A. W. (2000) J. Chem. Technol. Biotechnol. 75, 823–841
9. Guardiola-Diaz, H. M., Foster, L.-A., Mushrush, D., and Vaz, A. D. N. (2001) Biochem. Pharmacol. 61, 1463–1470
10. Munro, A. W., McLean, K. J., Marshall K. R., Warman, A. J., Lewis, G., Roitel, O., Sutcliffe, M. J., Kemp, C. A., Modi, S., Serotonin, N. S., and Leys, D. (2003) Biochem. Soc. Trans. 31, 625–630
11. McLean, K. J., Marshall, K. R. Richmond, A., Hunter, S. I., Fowler, K., Kieser, T., Gurcha, S. S., Besara, G. S., and Munro, A. W. (2002) Microbiology 148, 2937–2949
12. Lamb, D. C., Kelly, D. E., Baldwin, B. C., and Kelly, S. L. (2000) Chem. Biol. Interact. 125, 165–175
13. Lamb, D. C., Kelly, D. E., Schunk, W.-H., Shyadehi, A. Z., Aktar, M., Lowe, D. J., Baldwin, B. C., and Kelly, S. L. (1997) J. Biol. Chem. 272, 5682–5688
14. Lamb, D. C., Kelly, D. E., Waterman, R. M., Strumme, M., Rozman, D., and Kelly, S. L. (1999) Yeast 15, 755–763
15. Dijamghanian, S., Gerber, J. G., Sanchez, A., and Gal, J. (2004) Chirality 16, 79–85
16. Georgopapadakou, N. H. (1998) Curr. Opin. Microbiol. 1, 547–557
17. Sheehan, D. J., Hightock, C. A., and Sibley, C. M. (1999) Clin. Microbiol. Rev. 12, 40–79
18. Domencich, B. (1999) Curr. Opin. Microbiol. 2, 509–515
19. Lamb, D. C., Kelly, D. E., Baldwin, B. C., Gasso, F., Boscott, P., Richards, W. G., and Kelly, S. L. (1997) FEMS Microbiol. Lett. 149, 25–30
20. Ji, H., Zhang, W., Zhang, M., Kudo, M., Aoyama, Y., Yoshida, Y., Sheng, C., Song, Y., Yang, S., Zhou, Y., Lu, J., and Zhu, J. (2000) J. Med. Chem. 43, 4745–4755
21. Cabello-Hurtado, F., Taton, M., Forthoffer, N., Kahn, R., Bak, S., Rahier, A., and Weckreich-Reichhart, D. (1999) Eur. J. Biochem. 262, 435–446
22. Podust, L., Pouls, T. L., and Waterman, M. R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3070–3075
23. Podust, L. M., Stojan, J., Pouls, T. L., and Waterman, M. R. (2001) J. Inorg. Biochem. 87, 227–235
24. Zarn, J., J., Brandi, B. J., and Schlatter, J. R. (2003) Environ. Health Perspect. 111, 255–261
25. Pouls, T. L., and Howard, A. J. (1987) Biochemistry 26, 8165–8174
26. Bellamine, A., Mangla, A. T., Nes, W. D., and Waterman, M. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8937–8942
27. Lepesheva, G. I., Virus, C., and Waterman, M. R. (2003) Biochemistry 42, 9091–9101
28. Bellamine, A., Lepesheva, G. I., and Waterman, M. R. (2004) J. Lipid Res. 45, 2000–2007
29. Lepesheva, G. I., Nes, W. D., Zhou, W., Hill, G. C., and Waterman, M. R. (2004) Biochemistry 43, 10789–10799
30. Cosme, J., and Johnson, E. F. (2001) J. Biol. Chem. 275, 2545–2553
31. Cosme, J., Barnes, H. J., and Waterman, M. R. (1993) Arch. Biochem. Biophys. 304, 272–278
32. Wachenfeldt, C., Richardson, T. H., Cosme, J., and Johnson, E. F. (1997) Arch. Biochem. Biophys. 339, 107–114
33. Uchida, T., Mogi, T., Nakamura, H., and Kitagawa, T. (2004) J. Biol. Chem. 279, 53613–53620
Structural Diversities between Azole-bound Forms of CYP51

35. Lipscomb, J. D. (1980) Biochemistry 19, 3590–3599
36. Chen, Z., Ost, T. W. B., and Schelvis, P. M. (2004) Biochemistry 43, 1798–1808
37. Wells, A. V., Li, P., Champion, P. M., Martinis, S. A., and S Sixar, S. G. (1992) Biochemistry 31, 4384–4393
38. McLean, K. J., Cheesman, M. R., Rivers, S. L., Richmond, A., Leys, D., Chapman, S. K., Reid, G. A., Price, N. C., Kelly, S. M., Clarkson, J., Smith, W. E., and Munro, A. W. (2002) J. Inorg. Biochem. 91, 527–541
39. Deng, T-J., Proniewicz, L. M., Kineaid, J. R., Yeom, H., Macdonald, I. G., and S Sixar, S. G. (1999) Biochemistry 38, 10990–10996
40. Chen, Z., Wang, L. H., and Schelvis, P. M. (2003) Biochemistry 43, 1396–1403
41. Hu, S., Morris, I. K., Singh, J. P., Smith, K. M., and Spiro, T. G. (1993) J. Am. Chem. Soc. 115, 12446–12458
42. Smith, S. J., Munro, A. W., and Smith, W. E. (2003) Biopolymers 70, 620–627
43. Noble, W. A., Quaroni, L., Chumanov, G. D., Turner, K. L., Chapman, S. K., Hanzlik, R. P., and Munro, A. W. (1998) Biochemistry 37, 15799–15807
44. Kapetanaki, S., Chouchane, S., Giretto, S., Yu, S., Maglioza, R. S., and Schelvis, J. P. M. (2003) Biochemistry 43, 3835–3845
45. Peterson, E. S., Friedman, J. M., Chien, E. Y. T., and S Sixar, S. G. (1998) Biochemistry 37, 12301–12319
46. Dawson, J. H., Anderson, R. A., and Sono, M. (1982) J. Biol. Chem. 257, 3606–3617
47. Tsai, A.-L., Berka, V., Chen, P.-F., and Palmer, G. (1996) J. Biol. Chem. 271, 32563–32571
48. Berka, V., Palmer, G., Chen, P.-F., and Tsai, A.-L. (1998) Biochemistry 37, 6136–6144
49. Solomons, T. W. G. (1996) Organic Chemistry 6th ed., John Wiley and Sons, New York
50. Tanaka, T., Okuda, T., and Yamamoto, Y. (2004) Chem. Pharm. Bull. 52, 830–835
51. Williams, P. A., Casme, J., Ward, A., Angove, H. C., Vinkovnic, D. M., and Jhoti, H. (2003) Nature 424, 464–468
Structural Diversities of Active Site in Clinical Azole-bound Forms between Sterol 14 α-Demethylases (CYP51s) from Human and Mycobacterium tuberculosis
Koji Matsuura, Shiro Yoshioka, Takehiko Tosha, Hiroshi Hori, Koichiro Ishimori, Teizo Kitagawa, Isao Morishima, Norio Kagawa and Michael R. Waterman

J. Biol. Chem. 2005, 280:9088-9096.
doi: 10.1074/jbc.M413042200 originally published online December 20, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M413042200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 11 of which can be accessed free at http://www.jbc.org/content/280/10/9088.full.html#ref-list-1