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The Amino Acid Residues Affecting the Activity and Azole Susceptibility of Rat CYP51 (Sterol 14-Demethylase P450)

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The amino acid residues affecting the function of rat sterol 14-demethylase P450 (CYP51) were examined by means of point mutation. Forty-five mutants with respect to 27 amino acid sites were constructed and expressed in *Escherichia coli*. Substitution of highly conserved Y131, E369, R372, or R382 decreased the expression of CYP51 protein, indicating some structural importance of these residues. Substitution of H314, T315, or S316 caused considerable effects on the catalytic activity, and T315 was identified as the "conserved threonine" of CYP51. H314 was important for maintenance of the activity of CYP51 and was a characteristic residue of this P450, because the position corresponding to this residue is occupied by an acidic amino acid in most other P450 species. A144 was identified as a residue affecting the interaction of CYP51 with ketoconazole. Substitution of A144 with I, which occupies the corresponding position in fungal CYP51, enhanced the ketoconazole susceptibility of rat CYP51 with little change in the catalytic activity, indicating an important role of this residue in determination of the ketoconazole susceptibility of CYP51. Alteration of the catalytic activity was caused by the substitution at some other sites, whereas substitution of a few highly conserved amino acids caused little alteration of the activity of CYP51.

Key words: azole-antifungal agents, CYP51, moleculer modeling, point mutation, sterol 14-demethylase P450.

Sterol 14-demethylase (CYP51) is an essential enzyme that mediates the oxidative removal of the 14α-methyl groups of the precursors for sterol biosynthesis in eukaryotes (*1–3*). CYP51 is an exceptional P450 that has been conserved in the biological kingdoms (*4–6*). Although animal, plant, and fungal CYP51s are orthologous enzymes derived from a common ancestor and catalyze essentially the same 14-demethylation of sterol precursors (*4*, *6*), their substrate specificities are different (*6–10*), reflecting the different major sterols produced in each kingdom; cholesterol, phytosterols, and ergosterol in animals, plants, and fungi, respectively. The characteristic differences observed between the substrate specificities of these CYP51s seem to depend on their ability to recognize the peripheral structures of the substrate molecules, such as 4-methyl and 24-methylene groups of sterol substrates having the common 14-methylcholestene skeleton (*7–9*). This suggests that some local structural differences have occurred among the substrate-recognition sites of CYP51s of mammals, plants, and fungi.

CYP51 is the target of azole antifungal agents (*11–13*). However, the susceptibility to azole antifungal agents differs among fungal and mammalian CYP51s (*10*, *14*, *15*). Since azole antifungal agents are known to interact with both the heme iron and the substrate-recognition site or its vicinity of CYP51 (*16–18*), the structures necessary for interaction with azole compounds are probably different between mammalian and fungal CYP51s, although azole nitrogen commonly binds to the heme iron.

These facts suggest that some alterations of local structures that occurred in CYP51 after the separation of animals and fungi have modified the substrate specificity and azole susceptibility. As discussed extensively in our recent review (*6*), these alterations are interesting examples demonstrating the diversification of one distinctive P450 species during evolution. Then, we analyzed the local structures responsible for the activity and azole susceptibility of rat CYP51 by site-directed mutagenesis. In this work, we constructed 45 mutants with respect to 27 amino acid sites and expressed them in the membrane fraction of *Escherichia coli*, and then analyzed the effects of amino acid substitutions on lanosterol 14-demethylase activity and the susceptibility to ketoconazole, a typical azole antifungal agent, of the mutant CYP51s. The results showed that several amino acid residues around the 315th threonine included in one of the putative substrate-recognition sites (SRS-4), and some additional amino acid residues existing in other SRSs were important for the activity. It was also revealed that the 144th isoleucine was one of the amino acid residues affecting the azole susceptibility of CYP51.

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Abbreviations: CYP51, sterol 14-demethylase P450; IC_{50}, concentration for 50% inhibition.

MATERIALS AND METHODS

Chemicals, Biochemicals, and Host-Vector System—Ketoconazole was obtained from Sigma. NADPH-P450 reductase was purified from rat liver microsomes according to the method of Yasukochi and Masters (*19*). Other chemicals, biochemicals, and enzymes used in this study were guaranteed reagents obtained from commercial sources. The expression vector, *i.e.* the pCWori$^+$ plasmid, and the host (*E. coli* JM109) were kindly provided by Dr. M.R. Waterman.

Site-Directed Mutagenesis of Rat CYP51—Rat CYP51 cDNA was cloned with the expression plasmid pCWori$^+$ as described previously (*10*). In this plasmid the nucleotide sequence of CYP51 encoding the N-terminal 8 amino acid residues was modified to improve the expression efficiency in *E. coli*, and this modified rat CYP51 cDNA insert was called CYP51m, as described previously (*10*). The *Bam*HI–*Hin*dIII fragment, which covers the whole CYP51m cDNA insert from plasmid pCW$_{ratCYP51m}$ was subcloned into the *Bam*HI–*Hin*dIII site of pBluescript KS$^-$ to prepare the pBK$_{ratCYP51m}$ plasmid. Site-directed mutagenesis of rat CYP51 was performed with the thus prepared pBK$_{ratCYP51m}$ plasmid using a Transformer™ Site-Directed Mutagenesis Kit (CLONTECH Laboratories). The selection primer was the *Afl*III/*Bgl*II oligo primer (20 mer), which converts a unique *Afl*III site in the pBluescript KS$^-$ plasmid to a *Bgl*II site. The mutagenic primers (20 mer) having the nucleotide sequences that caused single amino acid substitutions at the desired sites in rat CYP51 were supplied by Sawady Technology (Tokyo). After confirmation of the nucleotide sequence of the resulting mutant CYP51m cDNA insert, the *Bam*HI–*Hin*dIII fragment from the modified pBK$_{ratCYP51m}$ was inserted into the *Bam*HI–*Hin*dIII site of pCWori$^+$ to construct an expression plasmid of the CYP51 mutant. Expression of CYP51 mutants in *E. coli* and preparation of a membrane fraction of *E. coli* were carried out by the previously described method (*10*). The membrane fraction was stored under N_2 at –80°C.

Analytical Methods—The P450 content of the membrane fraction of *E. coli* cells was determined spectrophotometrically using the extinction coefficient difference between 450 and 490 nm of the reduced CO-difference spectrum of 91.1 mM^{-1} cm^{-1} (*20*). Protein was determined by the method of Lowry *et al.* (*21*) with bovine serum albumin as the standard.

Assaying of Enzymatic Activity—The sterol 14-demethylase activity of the CYP51 mutants expressed in the *E. coli* membrane fraction was assayed with lanosterol as the substrate in the presence of purified rat liver NADPH-P450 reductase as described previously (*7*, *10*). The reaction mixture (1.0 ml) comprised the membrane fraction containing 0.1 nmol CYP51, 0.04 unit NADPH-P450 reductase, 25 nmol lanosterol dispersed in Tween 80, 50 mM potassium phosphate buffer, pH 7.5, and 0.15 mM NADPH with its generator (glucose 6-phosphate and glucose-6-phosphate dehydrogenase). The reaction was conducted aerobically at 37°C for 15 min, and the activity was calculated from the initial amount of the substrate, lanosterol, and the gas-chromatographically determined conversion ratio of the substrate to the 14-demethylated metabolite (*1*). Ketoconazole was added to the reaction mixture as a dimethylsulfoxide solution.

Molecular Modeling of Rat CYP51—Molecular modeling of rat CYP51 was performed as described by Yabusaki *et al.* (*22*) using the crystallographic structure of CYP102 (*23*, PDB; 2HPD) as a template. The amino acid sequences of CYP51 and CYP102 were aligned by means of the most recent algorithm of Gotoh (*24*), and model construction and energy minimization were performed using QUANTA/CHARMm (MSI) run on an IRIS-Crimson/Elan workstation (SGI). Further details of the model construction will be presented elsewhere.

RESULTS AND DISCUSSION

Selection of Target Amino Acid Residues Based on Amino Acid Sequence Alignment and Computer-Aided Modeling—CYP51 is a structurally and functionally conserved P450 species showing a wide distribution in the biological world (*4–6*). Amino acid sequence alignment of all known CYP51 sequences demonstrated the occurrence of six conspicuously conserved regions, most which overlap the putative

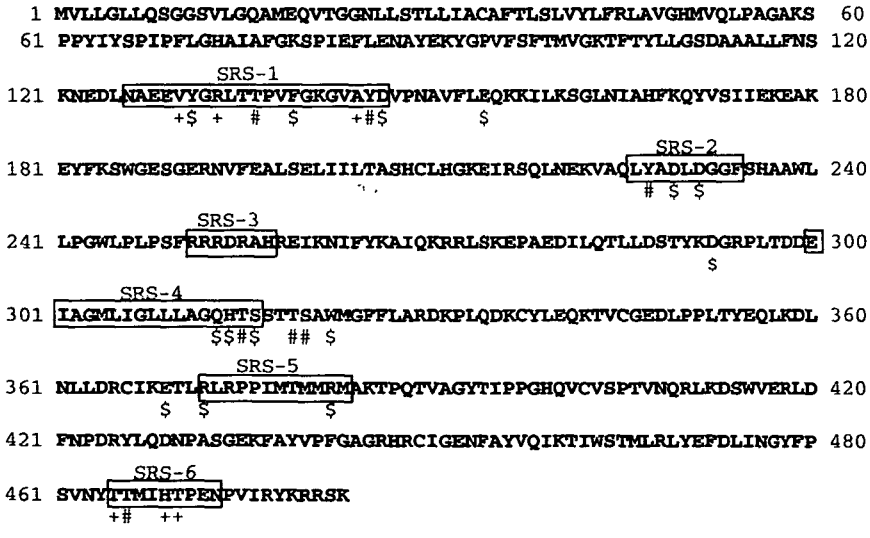

Fig. 1. **Amino acid residues selected as the targets of substitution.** The target amino acid residues are denoted by $, #, or +. The amino acids denoted by $, #, or + are those conserved in all eukaryotic CYP51s, those conserved in animal and most fungal ones, and those conserved in only animal ones, respectively. The regions corresponding to the putative substrate-recognition sites, SRS-1 through 6, are boxed.

substrate-recognition sites (SRSs) and the heme-binding domain (*4–6*). This strongly suggests that the amino acid residues included in these conserved regions are important for maintenance of the function of CYP51. In this study, we selected 27 amino acid residues occurring in the SRSs and their vicinity as the targets of the amino acid substitution experiment, as illustrated in Fig. 1. Most of the substituted amino acid residues are conserved ones in eukaryotic CYP51s. The functionally apparent heme-binding domain and low conservational SRS-3 were omitted from the target.

The adequacy of the selected amino acid residues for examining the structure and function relationship of CYP51 was assessed with a three-dimensional model of rat CYP51 constructed using CYP102 (*23*) as the template (Fig. 2). As shown in Fig. 2, the shape of the model of rat CYP51 was typical of P450 (*23*, *25–28*), and the helical regions considered to be important for maintaining the function of P450 (*25*), such as helices B′, F, G, and I, were located in reasonable places. The six SRSs (in different colors in Fig. 2) predicted on amino acid sequence alignment (*4*, *5*) were situated around the large cavity over the distal surface of the heme, and the space of this cavity was enough to accommodate a lanosterol molecule (Fig. 2). These facts supported the adequacy of the prediction of SRSs and the selection of the amino acid residues to be substituted, as illustrated in Fig. 1.

Mutations Decreasing the Expression of CYP51 Protein in E. coli—Forty-five rat CYP51 point mutants with respect to 27 sites (Fig. 1, and Tables I and II) were expressed in *E. coli* cells. Most of the mutants showed significant expression, but the Y131S, E369A, R372A, and R382A mutants gave no spectrophotometrically detectable P450 (Table II). These 4 mutant CYP51 genes produced no protein reacting with anti–rat CYP51 antibodies (*10*) on Western blotting (data not shown), indicating that the mutations at these sites decreased the production of CYP51 protein itself or that these mutant proteins might be quite unstable in the host cells. The 131st Y is conserved in all known CYP51s and is situated in the middle of SRS-1 (see Fig. 5). E369, R372, and R382 are also conserved in all known CYP51s, and are located in SRS-5 and its vicinity. These facts suggest essential roles of these amino acid residues in CYP51 expression. Amino acid sequence alignment has suggested that E369 and R372 correspond to those of the conserved E-X-X-R motif in the K helix (*29*), which was considered to be important for heme holding (*29*, *30*). Accordingly, the lack of expression of the E369A and R372A mutants seems to suggest their contribution to the same function as above. However, further details of the structural roles of these residues have not yet been revealed.

Effects of Amino Acid Substitution in the I-Helix Region on the Catalytic Activity—Table I summarizes the results of amino acid substitutions in the putative I-helix region assumed from the amino acid sequence alignment and supported by the molecular modeling (Fig. 2). The I-helix is located over the distal side of the heme group as in the case of other P450 species (*23*, *25–28*), and the putative I-helix region was also situated at the same place in the three-dimensional model of rat CYP51 (Fig. 2). The relative location of this region as to the heme in CYP51 was confirmed on spectrophotometric analysis of the T315K mutant. As shown in Fig. 3A, the Soret band of this mutant CYP51 showed marked a red shift from that of the native form, indicating elevation of the field strength of the axial ligand of the heme iron. It was also found that this mutant showed no spectrophotometrically detectable interaction with

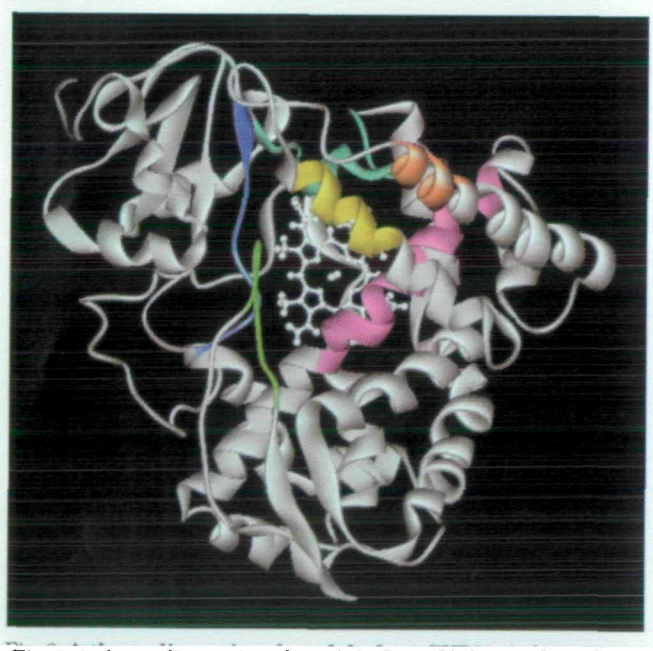

Fig. 2. **A three-dimensional model of rat CYP51.** A three-dimensional model of rat CYP51 was constructed using the crystallographic structure of CYP102 as the template. The N-terminal 54 amino acid residues of rat CYP51 that had no corresponding residues in the CYP102 sequence were removed upon the model construction. The regions assignable as the putative substrate-recognition sites are indicated by different colors, *i.e.* cyan (SRS-1), yellow (SRS-2), amber (SRS-3), magenta (SRS-4), blue (SRS-5), and green (SRS-6). The heme is shown as a white ball and stick model.

TABLE I. **P450 yields and lanosterol 14-demethylase activities of the mutants with respect to the amino acids in the I helix region.** The P450 yield and lanosterol 14-demethylase activity were measured as described under "MATERIALS AND METHODS." The P450 yields shown in this table are representative of two independent expression experiments. The lanosterol demethylase activity values are the means of three measurements for each specimen.

Mutant	P450 yield (nmol/100 ml culture)	Lanosterol demethylase activity (nmol/min/nmol P450)	(%)
Wild type	13.2	1.29 ± 0.29	100
E300A	4.2	1.58 ± 0.79	122
Q313A	9.6	1.05 ± 0.16	81.4
H314F	8.4	0.55 ± 0.13	42.6
H314A	16.6	0.45 ± 0.19	34.9
H314K	17.3	0.26 ± 0.05	20.2
H314D	22.3	0.18 ± 0.01	14.0
T315S	14.3	0.98 ± 0.27	76.0
T315A	12.6	0.22 ± 0.08	17.1
T315V	8.8	< 0.1	< 8
T315K	14.0	< 0.1	< 8
T315N	15.4	< 0.1	< 8
S316A	15.3	1.07 ± 0.09	82.9
S316T	38.7	0.64 ± 0.08	49.6
S316V	32.4	0.52 ± 0.14	40.3
S316L	25.3	0.25 ± 0.05	19.4
T319A	11.7	1.06 ± 0.18	82.2
S320A	34.4	0.98 ± 0.19	76.0
W322F	5.0	0.98 ± 0.11	76.0

ketoconazole, which is known to interact with the 6th coordination position of the heme iron with the azole nitrogen (Fig. 3B). This indicated that the lysine residue interferes with the binding of the azole group of ketoconazole to the heme iron. These lines of evidence indicate that the ε-amino group of the substituted lysine residue interacts with the heme iron at the 6th coordination position, as in the case of the T301K mutant of CYP2C2 (*31*). Furthermore, the molecular model indicates the interaction of the ε-amino nitrogen of the lysine residue of the T315K mutant to the heme iron (Fig. 4). Thus, the I-helix region deduced on amino acid sequence alignment and molecular modeling is actually situated at the expected position in the CYP51 molecule, and T315 must be assignable as the so-called "conserved threonine" (*23*, *25*, *26*, *28*) corresponding to T252 of CYP101.

As discussed above, T315 is assigned as the conserved threonine of CYP51, and a "HTS" motif around this is conserved in most CYP51s, the exceptions being two fungal orthologues with a "HSS" motif. Substitution of these three amino acid residues, *i.e.* H314, T315, and S316, with other amino acids caused significant alterations of the activity (Table I). The conserved threonine is considered to play an important role in the oxygen activation by P450 (*32–34*). Substitution of T315 with other amino acids, such as A, V, K, and N, reduced the lanosterol 14-demethylase activity of CYP51 to less than 20% of that of the native enzyme (Table I). In contrast, the T315S mutant showed 76% of the normal activity, and this substitution has been found in two fungal CYP51s. These results suggest the necessity of an oxyamino acid at this site for maintenance of the activity of CYP51. This conclusion is in good agreement with the commonly accepted role of the conserved threonine in the catalytic function of P450 (*32–34*).

H314, which is conserved in all CYP51s, seems to be a residue characteristic of CYP51, because in many P450 species this position, *i.e.* the upstream neighbor of the conserved threonine, is occupied by an acidic amino acid. This acidic amino acid residue, D251 of CYP101 for example, is considered to play an important role in the proton transfer necessary for the formation of activated oxygen through the reduction of the oxygenated form of P450 by the second electron (*35–38*). This conclusion was made on the basis of analysis of the catalytic properties of CYP101 mutants having various amino acid substitutions at D251 (*35–38*). The necessity of an acidic amino acid at this position was supported by the point mutation experiment involving CYP1A2 (*39*). In the case of CYP51, however, substitution of H314 with D markedly reduced the activity (Table I). On the other hand, the H314F, H314A, and H314K mutants showed significant activity, the highest activity being observed for the H314F mutant (Table I). These findings suggest that the existence of an aromatic amino acid at this position is favorable for the catalytic activity of CYP51, but occupation of this position by an acidic amino acid is unfavorable, unlike in many other P450 species (*35–39*). It can thus be concluded that the existence of histidine as the

TABLE II. **P450 yields and lanosterol 14-demethylase activities of the mutants with respect to other regions.** The P450 yield and lanosterol 14-demethylase activity were measured as described under "MATERIALS AND METHODS." The P450 yields shown in this table are representative of two independent expression experiments. The lanosterol demethylase activity values are the means of three measurements for each specimen.

Mutant	P450 yield (nmol/100 ml culture)	Lanosterol demethylase activity (nmol/min/nmol P450)	(%)
Wild type	13.2	1.29 ± 0.29	100
V130A	3.3	0.87 ± 0.06	67.4
Y131F	1.1	trace	–
Y131S	0	–	–
R133G	7.7	1.24 ± 0.09	96.1
T136A	20.0	1.08 ± 0.18	83.7
T136S	30.8	1.49 ± 0.41	115
T136V	31.9	0.86 ± 0.10	66.7
F139A	4.2	0.19 ± 0.03	14.7
A144I	14.5	0.92 ± 0.19	71.3
A144V	21.0	0.88 ± 0.07	68.2
Y145A	16.5	1.26 ± 0.16	97.7
D146A	12.9	1.37 ± 0.21	106
E154A	5.4	1.23 ± 0.04	95.3
Y227A	9.3	0.72 ± 0.30	55.8
Y227F	25.5	1.07 ± 0.05	82.9
D229A	8.8	1.21 ± 0.23	93.8
D229V	18.1	1.12 ± 0.14	86.8
D229E	22.7	1.16 ± 0.11	89.9
D231A	17.9	0.37 ± 0.08	28.7
D231E	21.8	0.52 ± 0.08	40.3
E369A	0	–	–
R372A	0	–	–
R382A	0	–	–
T485A	18.1	1.20 ± 0.31	93.0
T486A	20.6	1.10 ± 0.49	85.3
H489A	9.1	1.52 ± 0.50	118
T490A	6.3	1.25 ± 0.56	96.9

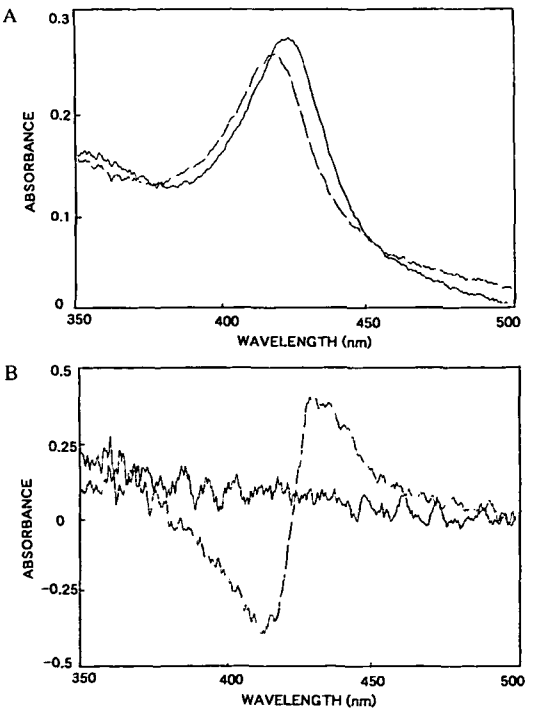

Fig. 3. **Spectral properties of the native form and T315K mutant of rat CYP51 solubilized from the membrane fraction of *E. coli*.** Membrane fractions of *E. coli* cells expressing the T315K mutant and native form of rat CYP51 were solubilized with 1% sodium cholate and then solubilized supernatants were prepared by centrifugation at 146,000 ×*g* for 120 min. A: Absolute spectra of the oxidized form of the native form (broken line) and the T315K mutant (solid line). B: Ketoconazole-induced difference spectra of the native form (broken line) and the T315K mutant (solid line).

upstream neighbor of the conserved threonine is important for CYP51, and that this is one of the structural characteristics of this P450 species. CYP51 mediates the C-C bond cleavage, and the active oxygen species used in this reaction is assumed to be peroxide on the heme iron (*2*, *40*). Since the aspartic acid residue adjacent to the conserved threonine in CYP101 is assumed to participate in the formation of an active single oxygen on the heme iron (*35–38*), the existence of histidine instead of an acidic amino acid at this position in CYP51 may be related to this unique reaction mechanism of this P450. However, the corresponding position of CYP19, which catalyzes similar C-C bond cleavage during the aromatization of androgens (*41*), is occupied by aspartic acid (*42*, *43*). Determination of the role of this residue in CYP51 would be interesting.

S316, the third residue of the HTS triplet, is also conserved in all CYP51s. Substitution of this residue with larger homologue threonine reduced the activity, and similar effects were also observed for the S316V and S316L mutants (Table I). In contrast, the S316A mutant showed nearly the same activity as the native enzyme (Table I). These facts suggest that the existence of serine at the 316th position may not be essential for the activity of CYP51 in spite of its high conservation, but its substitution with a larger amino acid may cause some steric hindrance as to substrate. Q313, which is the upstream neighbor of the HTS triplet, is also conserved in all CYP51s. However, substitution of this residue with alanine caused only a little reduction of the activity (Table I), indicating glutamine is not essential at this position for maintenance of the catalytic activity. These facts suggest the contribution of amino acids H314 and T315 to the catalytic activity of CYP51, but the role of each residue has not yet been clarified.

The existence of a 3β-hydroxyl group is essential for the substrates of CYP51 (*44*). This indicates that this hydroxyl group of the substrate may interact with a certain amino acid residue(s) of the enzyme, and identification of the residue(s) responsible for this function would be interesting. One candidate is T319 corresponding to T315 of *C. albicans* CYP51, which has been suggested to be the residue forming a hydrogen bond with the 3β-hydroxyl group of the sterol by Kelly's group based on the effect of a threonine to alanine substitution at this position (*45*). However, the substitution of T319 of rat CYP51 with alanine had no effect on the activity (Table I). Then, we substituted other possible candidates in the I-helix region, E300, S320, and W322, but none of them caused remarkable reduction of the catalytic activity (Table I), although E300 and W322 are conserved in all CYP51s. Thus, the amino acid residue responsible for the binding with the 3β-hydroxyl group of the substrate could not be identified in the I-helix region.

Effects of Amino Acid Substitution in Other Regions on the Catalytic Activity—Table II summarizes the effects of amino acid substitution in SRS-1, 2, 5, and 6, and their vicinity of rat CYP51. The most striking effect was observed on the substitution of highly conserved Y131 in SRS-1. Substitution of this residue with phenylalanine caused the complete loss of the activity and significant reduction of the expression level (Table II). The Y131S mutant was not expressed, as mentioned in the first section. These facts indicate that the 131st tyrosine is one of the key residues of CYP51 and that the function of this residue cannot be performed even with aromatic phenylalanine or serine having a hydroxyl group.

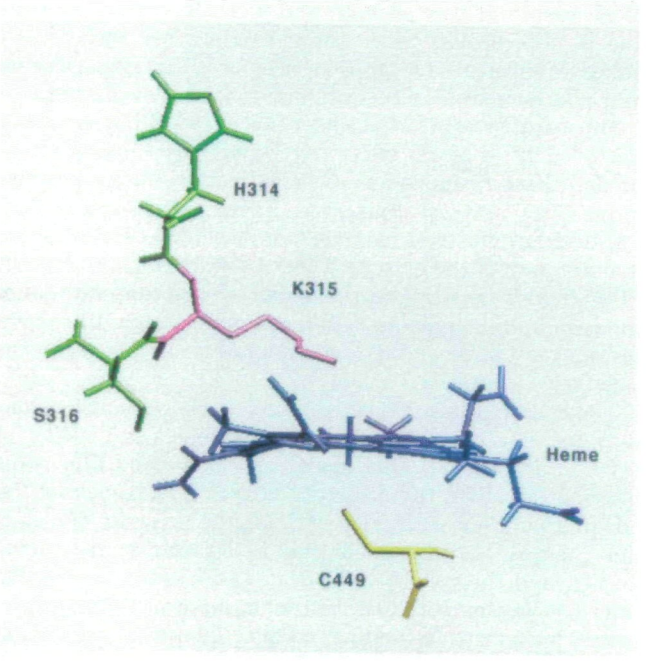

Fig. 4. **A three-dimensional model illustrating the interaction between the heme iron and the ε-amino group of the 315th lysine in the T315K mutant.** The 315th threonine of the three-dimensional model of rat CYP51 shown in Fig. 2 was replaced with lysine and then possible configurations of the lysine residue were calculated. This figure represents one of these possible configurations showing the interaction of the ε-amino group of the lysine residue (magenta) with the 6th coordination position of the heme (blue). The 449th cysteine providing the thiolate 5th ligand of the heme is indicated in yellow, and the 314th histidine and 416th serine are indicated in green.

SRS-1

rat	118	FNSKNEDLNAEE**VY**G**R**L**T**T**P**V**F**GKGV**AYD**VPNAVFL**E**QKKILKSGLNIA	166
human	118	FNSKNEDLNAEDVYSRLTTPVFGKGVAYDVPNPVFLEQKKMLKSGLNIA	166
C.albicans	105	FNAKLSDVSAEDAYKHLTTPVFGKGVIYDCPNSRLMEQKKFAKFALTTD	153
C.tropicalis	105	YNAKLSDVSAEEAYTHLTTPVFGKGVIYDCPNSRLMEQKKFAKFALTTD	153
S.cerevisiae	113	FNAKLADVSAEAAYAHLTTPVFGKGVIYDCPNSRLMEQKKFVKGALTKE	161
U.necator	109	LNGKLKDVNAEEIYTNLTTPVFGRDVVYDCPNSKLMEQKKFMKTALTIE	157
S.pombe	90	FNGKLADLNAEEAYSHLTTPVFGKDVVYDIPNHVFMEHKKFIKSGLGFS	138
U.maydis	117	FNAKHQQVTAEDAYTHLTTPVFGKEVVYDVPNAVFMEQKKFVKVGLSIE	165
S.bicolor	98	FKGNEAEMSQQEVY-RFNVPTFGPGVVFDVDYSVRQEQFRFFTEALRAN	145
A.thaliana	97	FKASESDLSQQEVY-QFNVPTFGPGVVFDVDYSVRQEQFRFFTEALRVN	144
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Fig. 5. **Amino acid sequence alignment of the region covering SRS-1 of CYP51.** This alignment was that extracted from the alignment of the entire amino acid sequences of the animal and fungal CYP51s described previously. The amino acid residues denoted by boldface letters in the rat sequence are those substituted in this work.

Substitution of F139 or D231 caused a more than 50% reduction of the catalytic activity (Table II). These residues are conserved in all CYP51s, indicating some important roles of these residues in the catalytic activity of this enzyme. Substitution of F139 with alanine caused marked reduction of the activity (Table II), suggesting that a small amino acid at this position is unfavorable. Substitution of D231 with alanine or glutamic acid had nearly the same effect on the catalytic activity (Table II). Therefore, aspartic acid itself may be required at this position and larger glutamic acid is unfavorable even though it is an acidic residue. In addition, substitution of Y227 with alanine caused a considerable decrease in the activity, whereas its substitution with phenylalanine little affected the activity (Table II), and in a few fungal CYP51s this position is occupied by phenylalanine. Accordingly, an aromatic amino acid must be necessary at this position and a small amino acid residue is unfavorable. Since these residues are included in putative substrate-recognition sites or their vicinity, they may play some role in the interaction with the substrate.

Substitution of R133, T136, Y145, D146, E154, or D229 caused a little or no effect on the activity (Table II), although these residues are included in the substrate-recognition sites and are conserved. These facts suggest that conserved amino acid residues are not always essential for maintenance of the activity. T485, T486, H489, and T490 in SRS-6 had little effect on the activity. Since conservation of the region assignable as SRS-6 was not so high, the above results may suggest the low potentiality of this region in substrate recognition.

The roles of the substituted amino acids were also examined using a computer-aided three-dimensional model of rat CYP51 (Fig. 2). This model provided some important information about the structure of rat CYP51, such as the adequacy of the assumption of SRSs, the presence of a suitable space for accommodating lanosterol in the heme pocket, and the role of T315 (Fig. 4). However, no reliable information was obtained for the amino acid residues necessary for substrate-binding, because lanosterol could dock in the heme pocket in several different orientations with its 14-methyl group in proximity to the oxygen bound to the heme iron.

Effects of Amino Acid Substitution on the Susceptibility to Ketoconazole—Azole antifungal agents are potent inhibitors of CYP51 (*11–13*). However, rat CYP51 shows relatively lower azole susceptibility than the fungal enzyme (*10, 14, 46*). Therefore, the effects of amino acid substitutions on the azole susceptibility were examined. V130 and A144 are interesting residues in this respect, because they are conserved in mammalian CYP51s but the positions corresponding to them are occupied by alanine and isoleucine or valine, respectively, in most fungal orthologues (Fig. 5). Substitution of A144 with isoleucine or valine increased the ketoconazole susceptibility of rat CYP51 (Fig. 6) with a little decrease in the activity (Table II). However, substitution of V130 with alanine did not alter the ketoconazole susceptibility of the enzyme. The apparent IC_{50} of ketoconazole as to the native rat CYP51 was 0.5 μM, but the IC_{50} was reduced to 0.1 μM in the A144I mutant (Fig. 6B). This indicates that replacement of A144 with isoleucine increased the affinity of rat CYP51 to ketoconazole, and this increase cannot be explained by the removal of steric hindrance. Consequently, the existence of isoleucine or valine at this position may be important for the interaction with ketoconazole, and the different ketoconazole susceptibilities observed for animal and fungal CYP51s must be due at least in part to the difference in the amino acid occupying this position, alanine for animal CYP51 and isoleucine or valine for fungal ones (Fig. 5).

In addition to the A144I and A144V mutants, the Y227A, H314A, and T315A ones showed higher ketoconazole susceptibility than the native form (Fig. 6A), but no mutant showed decreased susceptibility to the compound. Substitutions of these residues caused marked reduction of the activity, and ketoconazole susceptibility was elevated only when they were replaced with alanine. These results suggest that the latter three residues, *i.e.* Y227, H314, and T315, are responsible not only for substrate recognition but also for interaction with ketoconazole, and these residues may cause some steric hindrance with ketoconazole.

Recently, Asai *et al.* (*47*) reported that the mutations occurring in SRS-1 and its vicinity of *Candida albicans* CYP51 caused fluconazole resistance of CYP51. In this instance, the mutation was identified at Y132 of the *Candida* enzyme and this residue corresponds to Y145 of the rat enzyme (Fig. 5). However, substitution of this residue with alanine or histidine had no effect on the ketoconazole

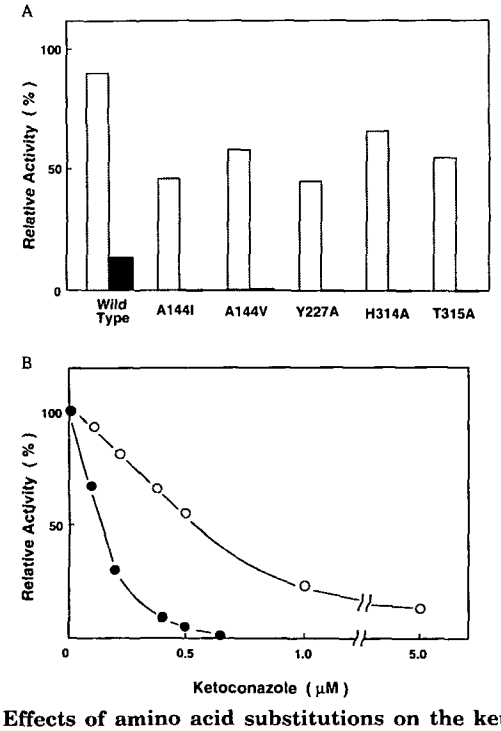

Fig. 6. **Effects of amino acid substitutions on the ketoconazole susceptibility of the lanosterol 14-demethylase activity of CYP51.** A: Lanosterol 14-demethylase activity of *E. coli* membrane fractions expressing the native from and the indicated point mutants of rat CYP51 were assayed in the presence of 0.1 μM (dotted bars) and 10 μM (closed bars) of ketoconazole, the activities being expressed as the values relative to those observed in the absence of ketoconazole. B: Effects of the ketoconazole concentration on the lanosterol 14-demethylase activity of the native form (open cirdes) and the A144I mutant (closed circles) of rat CYP51 expressed in the *E. coli* membrane fraction. The lanosterol 14-demethylase activity of rat CYP51 expressed in the *E. coli* membrane fraction was assayed in the presence of purified rat liver NADPH-P450 reductase as described under "MATERIALS AND METHODS."

susceptibility of the rat CYP51, although Y145H caused a significant decrease in the activity (data not shown). These observations constitute evidence for the interaction of azole antifungal agents with the substrate-recognition site and its vicinity of CYP51, which has been suggested by enzymatic experiments, and the contribution of specific amino acid residues to this interaction.

More recently, Waterman and associates succeeded in determination of the X-ray crystallographic structure of *Mycobacterium tuberculosis* CYP51 (M.R. Waterman, personal communication to Y.Y.). Although the function of CYP51 in the bacterium is not yet known, it can catalyze sterol 14-demethylation *in vitro* (*48*), and azole antifungal agents bind strongly to *M. tuberculosis* CYP51 (*49*). The structural homology of *M. tuberculosis* CYP51 to eukaryotic orthologues is not so high (*5*, *49*), but many identical amino acid residues have been identified in all CYP51s, and some of them were employed as the targets of amino acid substitutions in this work. Therefore, important information on the structure and function relationships of CYP51 will be obtained from the crystallographic structure of *M. tuberculosis* CYP51, and many uncertainties remaining after the present work will be explained by comparing the present results and the three-dimensional structure of *M. tuberculosis* CYP51.

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