[Frontiers in Bioscience 10, 1546-1557, May 1, 2005]

RECENT PROGRESS IN THE CYP51 RESEARCH FOCUSING ON ITS UNIQUE EVOLUTIONARY AND FUNCTIONAL CHARACTERISTICS AS A DIVERSOZYME P450

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1. ABSTRACT

CYP51 (sterol 14-demethylase P450) is a family of P450 species having the same function in distinct kingdoms, which makes CYP51 a unique and important enzyme family for the discussion of evolution and diversification of P450. Although CYP51 was named to the P450 originally discovered in yeast and fungi, today, we know that members of CYP51 family exist ubiquitously in animals, plants, fishes, lower eukaryotes such as slime mold, and a part of bacteria. In this review, I describe following subjects: 1) CYP51 as a model family for understanding of diversification of P450, 2) the structure and function relationships of CYP51s.

2. INTRODUCTION

Thousands of P450 genes in the huge *CYP* gene superfamily have been identified in all biological kingdoms. P450s are classified into hundreds of families, and in most cases, P450s belonging to an independent family are not distributed over different kingdoms. This fact suggests that various P450s occurring in current organisms may be differentiated and evolved independently after the establishment of major biological kingdoms. Actually, most of the functionally defined P450s are monooxygenases participating in peripheral and differentiated metabolisms of various organic compounds specific for the function of individual kingdoms or species. Such molecular and functional diversity is the most characteristic feature of P450, and a novel term "diversozyme" has been proposed for P450 by Coon (1).

CYP51 (sterol 14-demethylase: $P450_{14DM}$) is an exceptional P450 species distributing widely in eukaryotic kingdoms with the conserved metabolic function. CYP51 catalyzes the 14-demethylation of sterol precursors, and CYP51-dependent sterol 14-demethylation is included in all sterol biosynthetic pathways of eukaryotes (Figure 1) (2, 3). Furthermore, a P450 gene assignable as the member of CYP51 family was found in the genome of *Mycobacterium tuberculosis* (4, 5). Thus, CYP51 is considered to be a P450 species distributing in all biological kingdoms. This is the only known example of P450 family conserved throughout the evolution of eukaryotes. However, the substrate undergoing 14-demethylation in fungal, plant, and animal sterol biosynthetic pathways are different (Figure 1). Therefore, the occurrence of CYP51s showing different substrate specificities in fungi, plants, and animals can be considered as an example demonstrating the diversification of one distinctive P450 species into the enzymes showing different substrate specificities. Fungal CYP51 is the target of azole antifungal agents. Azole-resistant pathogenic fungi having azole-resistant CYP51s have been isolated (6, 7). Azole resistant CYP51s might be formed by mutations followed by selections through interaction with azole-rich environments. Accordingly, the examination on the mechanisms emerging azole-resistant CYP51 in fungi may provide a live example showing the mechanisms of P450 diversification. Consequently, CYP51 is the most suitable model for considering the molecular evolution and diversification processes of "diversozyme P450". The first section of this review discusses this topic.

The 14-demethylation of sterol precursors catalyzed by CYP51 is a complex reaction consisting of three successive monooxygenations (3, 8-10). To achieve C-C bond cleavage at the last step of these sequential reactions, critical geometry between the formyl-intermediate fixed at the active site and the molecular oxygen activated at the heme iron are required (Figure 2) (3, 10). Therefore, CYP51s must have a common or conserved structure necessary for supporting such complex reaction mechanism. Amino acid sequence identity of CYP51s occurring between different kingdoms is approximately 30 %, but a few conserved regions have been identified by amino acid sequence alignment (11). These findings suggest that the highly conserved regions

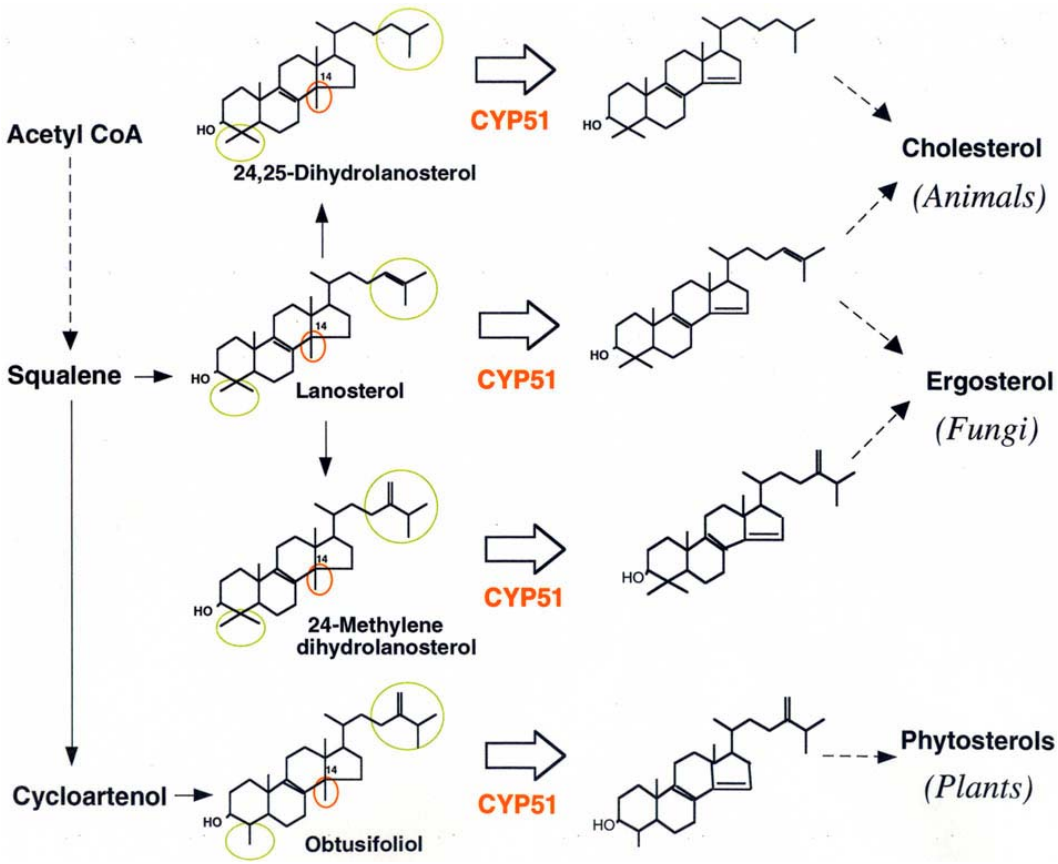

Figure 1. Sterol biosynthetic pathways of eukaryotes. The red circles indicate the 14-methyl group removed by CYP51. The local structures characteristic for the natural substrates of animal, plant, and fungal 14-demethylation steps are marked by green circles.

must be essential for supporting the unique reaction mechanism of this P450. Structural examination on CYP51 is expected to reveal the unique catalytic properties of CYP51, along with the elucidation of the structures responsible to the different substrate specificity of fungal, plant, and animal CYP51s, which may be an example of diversification of one distinctive P450 into the enzymes showing different substrate specificities. Structural studies of CYP51 have been carried out by site-directed mutagenesis and X-ray crystallography. X-ray crystallographic analysis was done on the *M. tuberculosis* CYP51 expressed in *Escherichia coli* (12). Site-directed mutagenesis was performed using rat CYP51 (13) as well as *M. tuberculosis* CYP51 (14, 15). The second section of this review deals with the recent progress of the structural studies of CYP51.

3. CYP51 IS AN EXAMPLE SHOWING THE DIVERSIFICATION PROCESS OF P450

3.1. Different substrate specificity of fungal, plant, and animal CYP51s is an example of P450 diversification

Sterol 14-demethylation is included in all sterol biosynthetic pathways of eukaryotes, which had been known as the P450-dependent reactions (3). Since the first finding of P450 responsible to this function as lanosterol 14-demetylase ($P450_{14DM}$, later named as CYP51) of yeast by Yoshida and his associates in the late 1970s (2, 16, 17), numbers of investigations have been done on this P450 species. In 1994, we reported that sterol 14-demethylase P450 of rat shows high amino acid sequence identity with yeast P45014DM (CYP51) (18) and suggested that yeast and rat P45014DM (CYP51) are orthologous enzymes conserved throughout eukaryotic evolution (11,□18). Furthermore, a gene (AC number; D55681) encoding a protein showing the highest amino acid sequence homology to CYP51 family was found in the genome of M. tuberculosis. The product of this gene expressed in E. coli showed the characteristics of CYP51 (4, 5). This finding showed the occurrence of CYP51 family in prokaryotic genome, and molecular phylogenetic analysis revealed that CYP51 appeared in the prokaryotic era and then distributed into all biological kingdoms through the divergence of life (19).

Amino acid sequence alignment of all known CYP51s indicates that the overall amino acid sequence identity between CYP51s of evolutionary distant species is about 30% (Table 1). However, amino acid sequences corresponding to the putative substrate recognition sites (SRSs) proposed by Gotoh (20) were outstandingly conserved among all CYP51s (Figure 3) (21). Such high structural conservation of SRSs in CYP51 makes marked contrast to the poorly conserved SRSs of CYP2 that is a family consisting of many typical diversozymes participating to the metabolism of various xenobiotics (21).

Table 1. Amino acid sequence identity between given pair of CYP51s from various species

Species	**Human**	**Rat**	***C. Albicans***	***S. Cerevisiae***	***U. Maydis***	***S. Pombe***	***D. Discoideum***	***S. Bicolor***	***A. Thaliana***	***M. Tuberculosis***
Human	100.0	93.4	38.0	36.6	38.4	39.7	34.5	35.4	37.7	33.3
Rat		100.0	38.4	35.7	39.3	39.9	34.3	36.0	38.3	32.0
C.albicans			100.0	64.8	45.6	46.8	27.2	27.8	26.3	37.8
S.Cerevisiae				100.0	47.5	49.3	30.7	29.7	30.7	28.1
U. Maydis					100.0	48.1	29.2	28.5	34.4	26.9
S. Pombe						100.0	32.3	30.4	30.9	30.4
D. Discoideum							100.0	38.6	39.5	30.5
S. Bicolor								100.0	76.1	33.8
A. Thaliana									100.0	37.9
M. Tuberculosis										100.0

Homology analysis of given pair of amino acid sequences was done by using the program of search homology included in GENETYX (Software Development Co., Tokyo).

The structural examinations with X-ray crystallographic analysis of CYP2C5 (22) and CYP2C9 (23) and site directed mutagenesis (24-27) revealed that the putative SRSs are actually located at the active-site responsible for the interaction with substrates. It can thus be concluded that CYP2 family has been diversified through alterations of the SRSs for adapting to metabolize wide variety of organic compounds. In contrast, CYP51 might have suppressed structural alterations of SRSs to conserve its essential role in sterol biogenesis by eukaryotes. However, it is noteworthy that substrates undergoing 14-demethylation in fungal, plant, and animal sterol biosynthetic pathways are different (Figure 1). This fact suggests a possibility that a slight difference may exist among substrate specificities of fungal, plant, and animal CYP51s, although amino acid sequences of their SRSs are highly conserved.

Examination was done on substrate specificities of rat and yeast CYP51s resulting in data that showed that 24,25-dihydrolanosterol and 24-methylenedihydrolanosterol were the specific substrates for rat and yeast CYP51s, respectively, although lanosterol was a good substrate for both of them (28). These substrate specificities of yeast and rat CYP51s correlate well with their intrinsic substrates in the sterol biosynthetic pathways (Figure 1). The sterol biosynthetic pathway of plants, whose natural substrate of CYP51 is obtusifoliol (Figure 1), is different from those of fungi and animals. CYP51s of maize, Sorghum bicolor, and wheat favorably metabolized obtusifoliol but showed no activity for lanosterol and 24,25-dihydrolanosterol (29-31). These facts clearly indicate that CYP51s of fungi, plants, and animals have different substrate specificities relating to the sterol precursors undergoing 14-demethylation in their sterol biosynthetic pathways. As discussed above, CYP51s occurring in all eukaryotes are orthologous P450 derived from a common ancestral species. Therefore, different substrate specificities observed among fungal, plant, and animal CYP51s suggests that development occurred independently on CYP51 of each kingdom. This may be a good example showing the differentiation of one distinctive P450 species into the different enzymes having modified substrate specificities.

3.2. Formation of *C. albicans* CYP51 variants through the interaction with environmental azole antifungal agents

Fungal CYP51 is the target of azole antifungal agent (32, 33). Azole resistant mutants of pathogenic *Candida albicans* have been isolated from patients receiving long-term medication with azole antifungal agents such as fluconazole (7, 34). Azole resistant *C. albicans* usually contained CYP51 variants showing reduced azole susceptibility (7, 34). Marichal *et al*. (6) reported that the amino acid substitutions of CYP51 variants occurring in azole-resistant *C. albicans* were frequently found in a few limited regions called "hot spot", suggesting that these regions might play some role upon interaction with azole compounds. This finding also suggested a possibility that fungal CYP51 underwent frequent mutation, but only the mutants having structural alterations in these regions have been selected under azole-rich environments. To obtain further evidence supporting this possibility, we have made extensive examination on an azole resistant CYP51 variant, $CYP51_{DUMC}$, occurring in an azole-resistant mutant of *C. albicans* strain DUMC136 in comparison with CYP51 of azole-sensitive standard strain of *C. albicans* ATCC 90028 (34, 35).

As described by Asai *et al*. (34), *CYP51* of *C. albicans* ATCC 90028 showed allelic variation having two amino acid substitutions at 116th and 128th positions. $CYP51_{DUMC}$ was derived from one of these alleles with two amino acid substitutions, Y132H and F145L (Figure 4A) (34). The region containing Y132 and F145 is corresponded to one of the hot spots of *C. albicans* CYP51 proposed by Marichal *et al.* (6). We then cloned and expressed *CYP51* alleles of *C. albicans* (named $CYP51_{ATCC\text{-}1}$ and $CYP51_{ATCC\text{-}2}$) and $CYP51_{DUMC}$ in *E. coli* and examined their catalytic activity and fluconazole sensitivity (35). No difference was observed between the fluconazole sensitivity of the allelic variants, $CYP51_{ATCC\text{-}1}$ and $CYP51_{ATCC\text{-}2}$. However, the fluconazole sensitivity of $CYP51_{DUMC}$ was remarkably low to compare with those of $CYP51_{ATCC\text{-}1}$ and $CYP51_{ATCC\text{-}2}$ (Figure 4B) (35). These findings clearly indicated that the *CYP51* variant encoding fluconazole resistant $CYP51_{DUMC}$ was selected under fluconazole-rich environments to emerge the fluconazole-resistant mutant *C. albicans* DUMC136, whereas allelic variants occurring in a wild-type strain *C. albicans* ATCC 90028 were both sensitive to fluconazole.

Using the purified preparations of CYP51

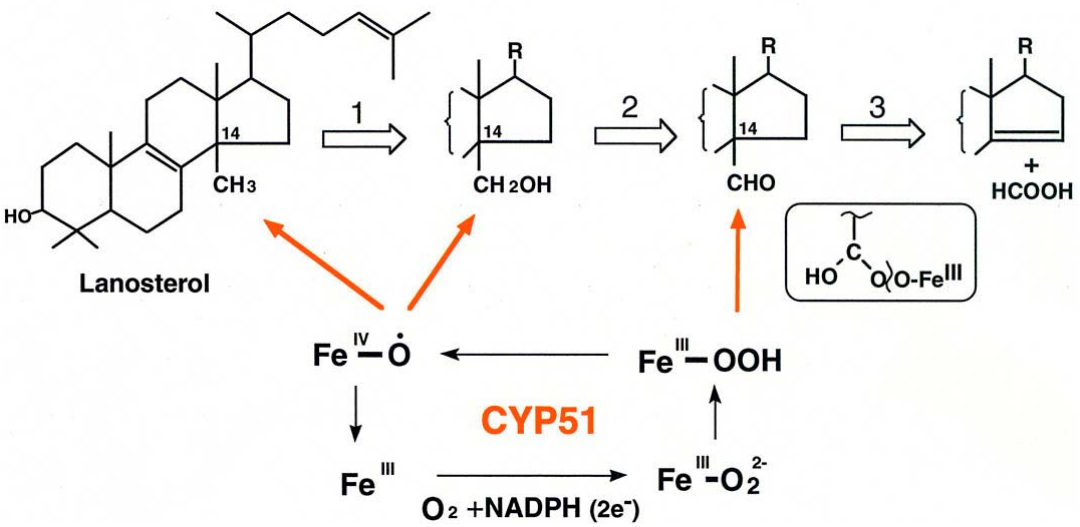

Figure 2. CYP51-mediated sterol 14-demethylation reaction consisting of three successive monooxygenations and the active oxygen species responsible to each monooxygenation step. The first and the second steps are ordinary monooxygenation driven by single oxygen activated at the heme iron. However, the last step is driven by the attack of peroxide on the heme iron. C-C bond fission is achieved by cleavage of peroxide linkage of the intermediate as shown in the box.

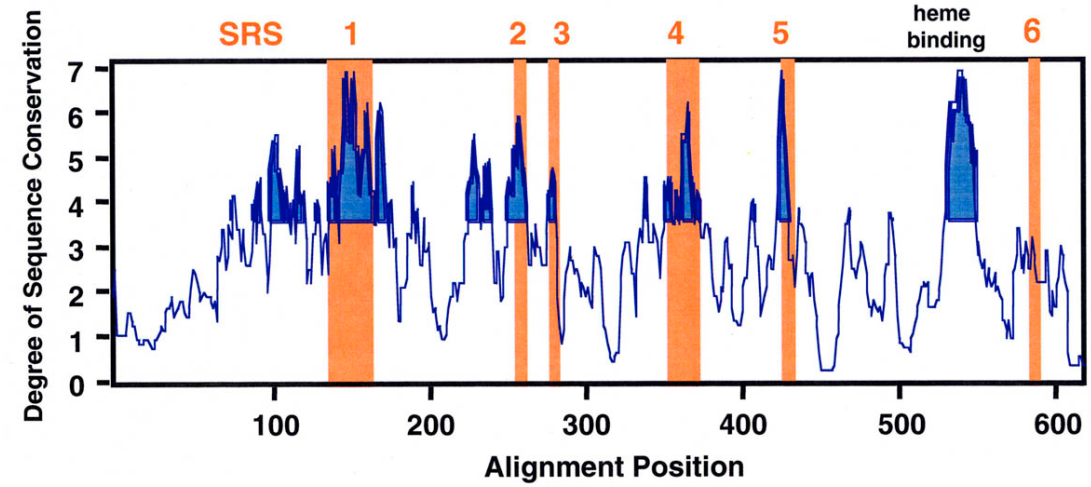

Figure 3. The relationship between the highly conserved region of CYP51s and SRSs of P450. All known amino acid sequences of eukaryotic CYP51s are aligned. The degree of sequence conservation at each alignment position was calculated as described previously (20). The regions denoted as SRSs 1 through 6 are corresponding to the putative substrate-recognition sites proposed by Goto (20). This figure was modified from that in Ref. 21

variants having Y132H and/or F145L substitutions, further detailed studies of these amino acid substitutions in enzymatic properties of CYP51 have been carried out (Kudo, M., Ohi, M, Aoyama, Y. and Yoshida, Y., 7th International symposium on cytochrome P450 biodiversity and biotechnology, Hyogo, Japan, 2004 and submitted to *J. Biochem.*). Important findings obtained in these studies are summarized as follows. 1) Y132H and F145L substitutions additively reduced the enzymatic activity of CYP51. 2) Introduction of both Y132H and F145L substitutions reduced the stability of CYP51 under reduced conditions. 3) Azole resistance of the variants having either Y132H or F145L substitution showed difference depending on azole compound. However, $CYP51_{DUMC}$ having both Y132H and F145L substitutions showed high resistance to all azole compounds so far tested. These findings indicated that both Y132H and F145L substitutions are effective for azole resistance of CYP51, although the effect of each substitution was different depending on azole compounds. In other words, two amino acid substitutions occurring in CYP51DUMC emerged resistance of the enzyme to multiple azole compounds. It is noteworthy that Y132H was one of the most frequent amino acid substitutions found in CYP51 variants of azole resistant mutants of *C. albicans*. Therefore, the amino acid substitution of F145L might be introduced to the variant having Y132H. The resulting variant, $CYP51_{DUMC}$, was selected due to its more potent fluconazole resistance.

Taken these findings together, it can be assumed

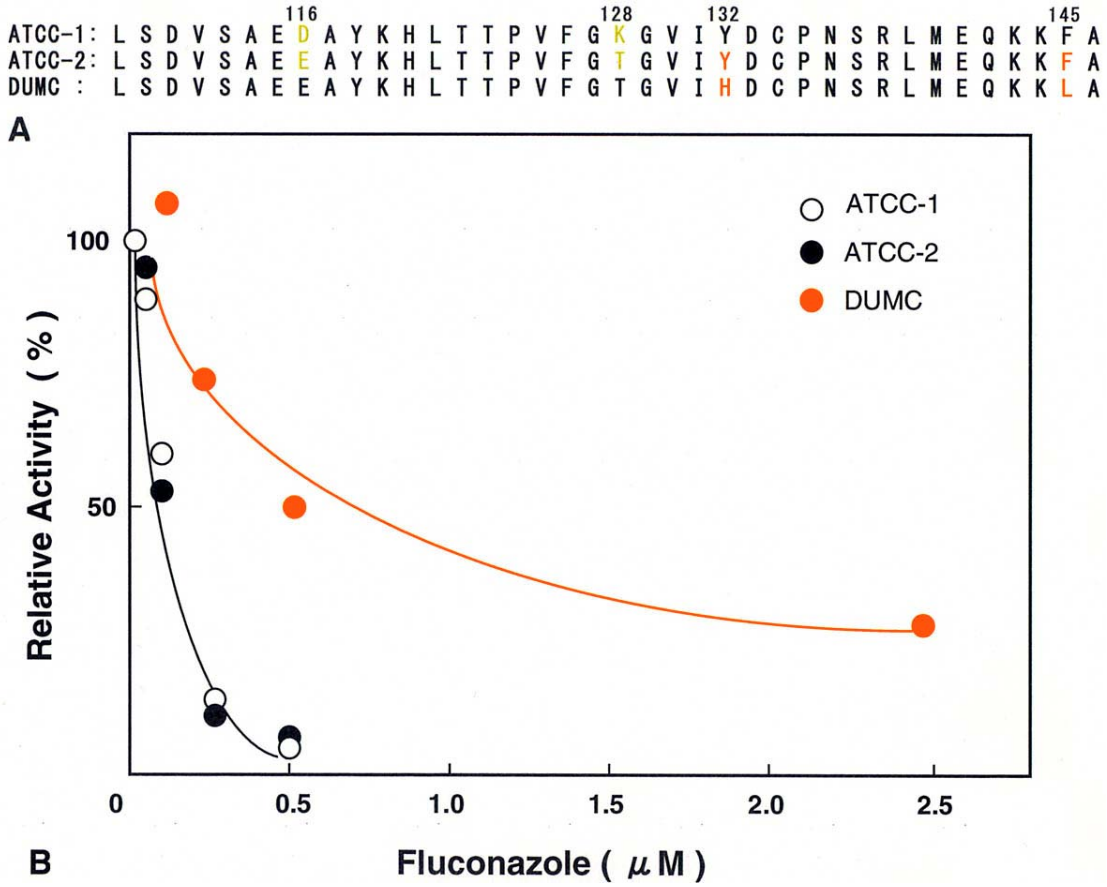

Figure 4. Fluconazole resistant CYP51 variants occurring in *C. albicans* strain DUMC136. Panel A: An amino acid sequence alignment of *C. albicans* CYP51 variants relating to fluconazole resistance. ATCC-1 and ATCC-2 are the allelic variants of CYP51 occurring in the fluconazole sensitive wild-type *C. albicans* ATCC 90028. DUMC is the fluconazole-resistant CYP51 variant emerged in the fluconazole resistant strain *C. albicans* DUMC136. Panel B: The inhibitory effects of fluconazole on lanosterol 14-demethylase activity of three CYP51 variants (35).

that *CYP51* of *C. albicans* undergoes frequent mutation, and the altered genes may be selected environmental pressure. Under normal environments, the variants retaining high activity must be selected as indicated by the occurrence of the allelic variants of *CYP51* in *C. albicans* ATCC 90028. However, under azole-rich environments, *CYP51* variant encoding an enzyme having decreased affinity to azole compounds may be selected even though its catalytic activity is considerably lower than the native enzyme. This is a good example demonstrating the formation of an altered P450 by mutation of an existing P450 species followed by the selection of the resulting variant through interaction with environmental chemicals. This is the core process of the possible mechanism of P450 diversification.

4. STRUCTURAL ANALYSIS OF CYP51

4.1. Structural examination of CYP51 with site-directed mutagenesis

Amino acid sequence alignment of CYP51s revealed that numbers of amino acid residues are conserved among them. Most of these conserved amino acid residues are found in putative SRSs and heme binding region. A computer-aided modeling of rat CYP51 with CYP102 as template indicated that most of these putative SRSs are located in the region around the heme pocket of the enzyme (13). The data suggested that the conserved amino acid residues in the putative SRSs might have structural importance for maintaining the characteristic catalytic function of CYP51.

To obtain the clues for solving this problem, Nitahara *et al.* (13) constructed 45 variants with respect to 27 conserved amino acid residues identified in the putative SRSs of rat CYP51 by site-directed mutagenesis. The effects of these amino acid substitutions on the function of the expressed proteins were examined in the study. The results obtained by these experiments are summarized in Table 2 (13). Remarkable effects of amino acid substitutions were observed on 9 positions: Y131, F139, D231, H314, T315, S316, E369, R372, and R382. Among these residues, Y131 in SRS-1 and E369, R372, and R382 in SRS-5 might be important for maintaining the structure, since substitution of any one of these residues diminished the expression of the proteins. The other five residues,

Table 2. Yields and lanosterol 14-demethylase activities of artificial CYP51 variants expressed in *E. coli* cells

Mutant	P450 Yield (nmol/100mL Culture)	Lanosterol 14-Demethylase Activity	
		(nmol/min/nmolP450)	(%)
Wild Type	13.2	1.29±0.29	100
Y131F	1.1	trace	-
Y131S	0	-	-
F139A	4.2	0.19±0.03	14.7
D146A	12.9	1.37±0.21	106
D231A	17.9	0.37±0.08	28.7
D231E	21.8	0.52±0.08	40.3
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
E369A	0	-	-
R372A	0	-	-
R382A	0	-	-

The amount of CYP51 variants expressed in the membrane fraction of *E. coli* cells was determined spectrophotometrically. Lanosterol 14-demethylase activity was measured by adding the purified preparation of rat NADPH-P450 reductase to the membrane fraction containing expressed CYP51 variants.

F139 in SRS-1, D231 in SRS-2, and H314, T315, and S316 in SRS-4, play essential roles in the activity of the enzyme. The role of the triplet, 314-HTS-316, existing in SRS-4 is worth discussing. Amino acid sequence alignment and molecular modeling showed that the triplet HTS is situated in I-helix. T315 in the triplet is corresponded to T252 of CYP101 that has been called as "conserved threonine" (36, 37). Substitution of T315 of rat CYP51 with K induced a marked red-shift of the Soret band, suggesting the interaction of the ε-amino group of K with the heme iron at the 6th coordination position. This assumption was supported by the molecular modeling of the T315K variant (13). Furthermore, the T315K variant showed no spectrophotometrically detectable interaction with ketoconazole (13), which is known to interact with the 6th coordination position of the heme iron with its imidazole nitrogen. These findings confirmed that T315 of rat CYP51 situates close to the distal side of the heme iron, suggesting that this T may be the residue corresponding to the conserved threonine. In a few fungal CYP51s, this position is occupied with serine (Figure 4). Substitution of T315 of rat CYP51 with serine reduced the activity to 76% of the control (Table 2). However, its substitution with other amino acid, such as A, V, K or N caused marked impairment of the catalytic activity (Table 2). This fact suggested the necessity of a hydroxy-amino acid at this position for maintaining the function of CYP51.

H314 is conserved in all CYP51s (Figure 5). However, this upstream neighbor of the conserved threonine is occupied by acidic amino acid residues in most P450 species. This acidic amino acid residue, for example, D251 of CYP101, plays a key role in the proton transfer necessary for oxygen activation (38, 39). Substitution of H314 of rat CYP51 to D caused marked reduction of the enzyme activity (Table 1), suggesting that the occurrence of histidine is necessary for maintaining the active structure of CYP51. In this respect, the following data are worth discussing. X-ray crystallographic analysis revealed that the I-helix of *M. tuberculosis* CYP51 is disturbed at H259 in the middle part of the helix (12 and Figure 6), and the N-terminal part of the helix is bent away from the core part of the protein (12). As discussed below, this bending of I-helix is important for forming an additional access channel to the active site of CYP51 (12 and Figure 6). Since H314 of rat CYP51 is corresponded to H259 of *M. tuberculosis* CYP51, this histidine conserved within CYP51s at this position may be essential for the bending of I-helix, therefore maintaining the activity of CYP51. The bending of I-helix may generally occur in CYP51.

S316 is also conserved in all CYP51s as shown in Figure 5. Little activity change was observed upon substitution of this residue with alanine, whereas its substitution with more bulky amino acid, such as valine or leucine, caused considerable decrement of the activity. The activity of S316T variant was about 50 % of the control. Therefore, hydroxy amino acid is not necessary at this position, in spite of high conservation of this residue. However, some steric hindrance may occur between this

animals	305	LIGLLLAGQHTSSTTSAW	322	Human
	305	LIGLLLAGQHTSSTTSAW	322	Pig
	305	LIGLLLAGQHTSSTTSAW	322	Rat
	305	LIGLLLAGQHTSSTTSAW	322	Mouse
	297	LIGLLLAGQHTSSTTSAW	314	Zebrafish
fungi	311	LIGILMGGQHTSASTSAW	318	*C.albicans*
	311	LIGVLMGGQHTSASTSAW	318	*C.tropicalis*
	318	LIGVLMGGQHTSAATSAW	325	*S.cerevisiae*
	319	LIGVLMGGQHTSAATSAW	326	*C.glabrata*
	319	MIALLMAGQHTSSATSSW	336	*U.maydis*
	311	MIALLMAGQHTSSATSSW	328	*F.neoformans*
	300	MIALLMAGQHSSSSTSSW	317	*N.crassa*
	302	MIALLMAGQHSSSSTSSW	319	*U.necator*
	301	MIALLMAGQHSSSSSSSW	318	*M.acuformis*
	301	MIALLMAGQHSSSSSSSW	318	*T.yallundae*
	301	MIALLMAGQHSSSSTASW	318	*A.fumigatusB*
	283	MITLLMAGQHSSSSISAW	300	*A.nidulans*
	299	MITLLMAGQHSSSAISCW	316	*P.italicum*
	299	MITLLMAGQHSSASISCW	316	*P.digitatum*
	313	MIALLMAGQHTSSATGSW	330	*P.chrysosporium*
	282	MIALLMAGQHTSAATIVW	299	*S.pombe*
protozoa	270	MIGLLFAGQHTSSITLTY	287	*D.discoideum*
plants	283	LIAALFAGQHTSSITSTW	300	*S.bicolor*
	280	LIAALFAGQHTSSITSTW	297	*O.sativa*
	282	LIAALFAGQHTSSITSTW	299	*A.thaliana2*
	279	LIAALFAGQHTSSITSTW	296	*T.ovary*
bacteria	250	FISMMFAGHHTSSGTASW	267	*M.tuberculosis*
	251	FISLMFAGHHTSSGTASW	268	*M.smegmatis*

Figure 5. Amino acid sequences of the I-helix part of CYP51s from various origins. Note that a triplet "HTS" or "HSS" at the center of I-helix is conserved among all known CYP51s.

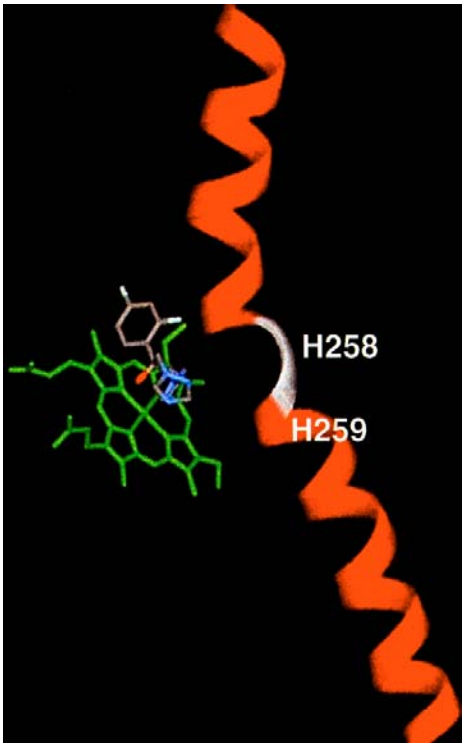

Figure 6. The I-helix bent at H258 and H259 of *M. tuberculosis* CYP51. The I-helix part was extracted together with the heme and fluconazole from the PDB file of the fluconazole-bound form of *M. tuberculosis* CYP51 (12).

residue and the substrate. Taken together, the HTS (or HSS) triplet in I-helix is a characteristic structural motif in CYP51 that discriminates it from other P450 species and plays important roles in maintaining the function.

Structural examination with site-directed mutagenesis was done also on *M. tuberculosis* CYP51. Lepesheva *et al*. constructed variants of *M. tuberculosis* CYP51 by substituting several amino acid residues conserved among CYP51s and examined the effects of amino acid substitutions on the catalytic activity and molecular properties of the enzyme (15). Substitution of Y76, F83, G84, D90, L172, G175, or R194 resulted in loss of the catalytic activity and diminished the Type I spectral change induced by the addition of 24, 25-dihydrolanosterol, suggesting their contribution to substrate binding (15). X-ray crystallographic analysis (see next section) showed that Y76 is one of the closest residues to the heme group, resulting in the formation of a hydrogen bond between its phenyl OH and the propionate side-chain of porphyrin A-ring. Since Y76F variant of *M. tuberculosis* CYP51 and the corresponding Y131F variant of human CYP51 showed no activity (15), this hydrogen bonding might be essential for the activity of CYP51. The expression of Y76F variant of *M. tuberculosis* CYP51 and Y131F variant of human CYP51 in *E. coli* was considerably decreased (15). Furthermore, Y131F variant of rat CYP51 was not expressed in *E. coli* (13). These results suggested that the above-mentioned hydrogen bonding between the conserved tyrosine and porphyrin A-ring is important for the stable expression of the heme protein.

In the three-dimensional structure of *M. tuberculosis* CYP51 (see next section), F83 is situated at the ceiling of the substrate-binding cavity above the heme plane. F83Y variant of *M. tuberculosis* CYP51 as well as the corresponding F139Y variant of human CYP51 showed no catalytic activity (15). F139A variant of rat CYP51 was expressed in a low level and showed markedly decreased activity (13). Therefore, this conserved phenylalanine is essential for maintaining the expression of the active forms of rat, human, and *M. tuberculosis* CYP51s.

The role of the conserved aspartate corresponding to D90 of *M. tuberculosis* CYP51 is worth discussing. D90 of *M. tuberculosis* CYP51 is included in the flexible BC loop and situates at the open part of the access channel (12). Lepesheva *et al*. suggested that D90 of *M. tuberculosis* CYP51 might move towards the inside of the molecule upon substrate binding and was likely to participate in substrate orientation during catalysis by forming a hydrogen bond with 3-OH of sterol (15). If this is the case, this residue plays a pivotal role for fixing the substrate at the active site during the catalytic cycle. However, D146A variants of human (15) and rat (13) CYP51 equivalent to the D90A variant of *M. tuberculosis* CYP51 showed the activity corresponding to 54 % and 106 %, respectively, of the activity of their parent forms. Consequently, the role of this conserved aspartate has not yet been confirmed.

As described at the beginning of this section, investigation of the roles of conserved amino acid residues by means of site-directed mutagenesis is one of the important approaches of structural study of CYP51. Lines of information indicating the essential roles of a few conserved amino acid residues have been obtained. However, many questions including the amino acid residues responsible to the substrate fixation to the active site remains unanswered.

4.2. The X-ray crystallographic analysis of *M. tuberculosis* CYP51

Important information for the structural feature of CYP51 has been obtained by the X-ray crystallographic examination of *M. tuberculosis* CYP51 expressed in *E. coli* (12). This CYP51 is a water soluble protein lacking the membrane-binding N-terminal domain. Although the function and natural substrates of this CYP51 have not yet been known, lines of evidence have shown structurally and functionally orthologous nature of this P450 to eukaryotic CYP51s (4, 5). As mentioned briefly in the preceding section, the most outstanding structural feature of *M. tuberculosis* CYP51 is the breaking of the long I-helix and resulting bent of the helix (12). In relation to this structural modification, BC loop has an open conformation to provide an additional access channel to the active site, leading to a structure having two access channels (12). Podust *et al.* assumed that these two channels might play different roles (12); one may be the entrance for substrate and the other might be the exit for metabolite. They also discussed a possibility of synchronized open and close movements of these two channels (12). This mechanism may enable the enzyme to make one-way flow from the substrate to the product through the active site. Since CYP51 mediates three successive monooxygenations without releasing intermediates to achieve C-C bond cleavage, such machinery may be important for this enzyme.

Based on the three-dimensional structure of *M. tuberculosis* CYP51 (12), Podust *et al.* discussed the structure responsible to the substrate recognition of CYP51 (40). Their approach was *in silico* docking of the substrate to the active site of *M. tuberculosis* CYP51 based on the assumption that the 14α-methyl group of lanosterol have to be near the oxygen molecule bound to the iron in order for the reaction to occur. Based on this simulation, they inferred that the regions corresponding to SRSs-4, 5, and 6, suggested by amino acid sequence alignment, were contributed to substrate binding. SRS-1 might make some interaction with the bound substrate when BC loop moved upon substrate binding. They also suggested that the regions corresponding to SRSs-2 and 3 did not contribute to the substrate recognition by CYP51. Such *in silico* simulation is an interesting approach for considering the structure responsible to substrate binding. However, amino acid sequence alignment indicated that SRSs-2 and 3 contains a few highly conserved amino acid residues. Substitutions of these residues caused considerable effects on the catalytic activity (13, 15). Accordingly, further studies must be done to reveal the structures necessary for determining substrate specificity of CYP51s. In this respect, the presentation by Podust *et al*. in the latest P450 biodiversity symposium held in Hyogo, Japan in August

2004 was interesting. They proposed that F78 might be a key residue in determining the substrate specificity of CYP51 for the sterols having 4-monomethyl-structure, such as obtusifoliol, or those having 4,4-dimethyl structure, such as lanosterol. This proposal is very interesting for considering the differentiation of the substrate specificity of CYP51 (see Figure 1). Amino acid residue corresponding to F78 of *M. tuberculosis* CYP51 is conserved as F among CYP51s of higher plants and bacteria, whereas the corresponding position is occupied by L among CYP51s of animals and fungi. CYP51 of higher plants metabolizes obtusifoliol as the most favorable substrate (Figure 1) (29-31). CYP51 of *M. tuberculosis* expressed in *E. coli* also metabolized obtusifoliol (5). On the other hand, fungal and animal CYP51s metabolize 4,4-dimethyl sterols such as lanosterol and 24-methylenedihydrolanosterol as natural substrates (Figure 1). The above-mentioned proposal by Podust *et al*. is seemingly coincided with these facts.

The X-ray crystallographic structure of *M. tuberculosis* CYP51 provided important information for considering structure and function relationship of CYP51. However, the structures necessary for determining the substrate specificity and fixing the intermediates at the critical position throughout the demethylation reaction consisting of three successive monooxygenations are yet to be clarified. To obtain necessary information for these considerations, X-ray crystallographic analysis of the substrate complexes of various CYP51s will be expected.

5. SUMMARY AND PERSPECTIVE

CYP51 is the only known P450 species distributing widely in all biological kingdoms with conserved metabolic function. However, some differentiations in substrate specificities have been found among animal, plant, and fungal CYP51s in relation to the difference in the sterol precursors undergoing 14-demethylation in each kingdom (Figure 1). This may be a good example showing the differentiation of one orthologous P450 species into a few different enzymes with modified substrate specificities. Recent progress in genome analysis of higher plants showed occurrence of multiple genes assignable as the members of CYP51 family (http://drnelson.utmem.edu/CytochromeP450.html). Intriguingly, over ten of putative *CYP51*genes are found in rice (*Oryza sativa*) genome (http://drnelson.utmem.edu/CytochromeP450.html). *CYP51* encoding sterol 14-demethylase is a single copy house-keeping gene in mammals. The house-keeping nature of *CYP51* must be retained in higher plants, because 14-demethylation is essential for the sterol biosynthesis also in higher plants. Therefore, occurrence of multiple genes belonging to CYP51 family in higher plant genome may suggest a possibility that some enzymes derived from CYP51 exist in higher plants. If this possibility is true, structural and functional examination of CYP51 family members of higher plants may provide interesting information for considering the mechanism of diversification of P450. *Arabidopsis thaliana* have two *CYP51* genes. One of them, named *CYP51A2,* has been identified as the gene encoding sterol 14-demethylase (41 and Aoyama *et al.*, unpublished data). Therefore, examination of the function of the enzyme encoded by another *CYP51* gene of *A. thaliana* (*CYP51A1*) has been expected to provide functional diversification of CYP51. However, Kim *et al*. communicated that *CYP51A1* of *A. thaliana* was a pseudogene (Kim *et al*., the 7th International symposium on cytochrome P450 biodiversity and biotechnology, Hyogo, Japan, 2004). We have obtained preliminary data leading us to the same conclusion by its expression in *E. coli*. Accordingly, functional diversification of plant *CYP51* remains to be an interesting problem to be solved, and functional characterization of multiple *CYP51* genes identified in rice genome is expected.

Sterols are not essential for bacteria. Therefore, intrinsic function of CYP51 occurring in *Mycobacterium* sp. has not yet been clarified, although *M. tuberculosis* CYP51 expressed in *E. coli* showed sterol 14-demethylase activity (5). Therefore, further examination to reveal the function of bacterial CYP51 is expected in order to understand the functional evolution of CYP51. In addition, Lamb *et al.* (42) suggested a possibility that one of the P450 genes occurring in *Streptomyces coelicolor* A3(2) genome might be a new member of CYP51 subfamily. They inferred this possibility based on the sterol 14-demethylase activity of the product of this gene expressed in *E. coli*. However, amino acid sequence alignment revealed that characteristic clusters constructing SRSs and heme-binding domain, which are conserved among the members of CYP51 family, were not found in the *S. coelicolor* P450. Therefore, this P450 species proposed as a new CYP51 by Lamb *et al*. (42) is not likely to be a member of CYP51 family, although it seemed to encode a P450 enzyme mediating 14-demethylation of sterol. If the 14-demethylase activity of this P450 reported by Lamb *et al*. (39) is not an artifact, this finding suggested the occurrence of another family of P450 having the catalytic property similar to CYP51. This is an interesting problem for considering functional evolution of P450. Further examination of the catalytic properties of this P450 is required.

CYP51 of mammals is an ubiquitously expressed single copy gene. The basic function of CYP51 must be the contribution to the *de novo* sterol synthesis in individual organs. However, there were several reports suggesting that ovarian CYP51 was responsible to the production of meiosis activating sterol, MAS (43, 44). This fact suggests a possibility that mammalian CYP51 has some organ specific functions in addition to the cholesterol synthesis. This may be another type of functional diversification of CYP51 observed specifically in multicellular organisms. CYP51 gene has multiple *cis*-regulatory elements in its promoter region, suggesting that expression of mammalian *CYP51* may be regulated differently among organs. In fact, expression of CYP51 in the liver and ovary of rat was dependent on different hormones (45, 46). Organ specific regulation of the CYP51 expression relating to the organ specific function is an interesting subject of CYP51 research.

Structural examinations with X-ray crystallographic analysis and site-directed mutagenesis of *M. tuberculosis* and a few mammalian CYP51s revealed the roles of several amino acid residues in the catalytic function of CYP51. However, the exact amino acid residues responsible to the substrate fixation at the active site are still uncertain. CYP51 mediates C-C bond cleavage reaction. This reaction is achieved by three successive monooxygenations keeping the substrate in the active site throughout the whole reaction process. Furthermore, critical interaction between the molecular oxygen activated at the heme iron and C_{32} of the substrate must be required at the third step of this reaction sequence to achieve C-C bond cleavage. Therefore, revelation of the structure necessary for such critical fixation of the substrate is very important for understanding the reaction mechanism of CYP51. To solve this important question, X-ray crystallographic analysis of the substrate complex of CYP51 is indispensable. For this purpose, crystallization of the substrate-bound eukaryotic CYP51 and/or intrinsic substrate of *M. tuberculosis* CYP51 is needed.

6. ACKNOWLEDGEMENTS

The author is deeply grateful to Dr. Norio Kagawa of Vanderbilt University School of Medicine for providing me an opportunity for preparing a review for Frontiers in Bioscience. The author was indebted to Dr. Yuzo Yoshida of Mukogawa women's university for his supervision, extensive discussion, and critical review for preparing this manuscript.

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Abbreviations: CYP51: sterol 14-demethylase, SRSs: substrate recognition sites

Key Words: CYP51, Sterol 14-demethylation, P450 evolution, P450 diversification, Structure and function, Cytochrome P450 Review

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