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Purification and Characterization of Rat Sterol 14-Demethylase P450 (CYP51) Expressed in *Escherichia coli*

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Sterol 14-demethylase P450 (CYP51) is an essential enzyme for sterol biosynthesis by eukaryotes. We have cloned rat and human CYP51 cDNAs [Aoyama, Y., Noshiro, M., Gotoh, O., Imaoka, S., Funae, Y., Kurosawa, N., Horiuchi, T., and Yoshida, Y. (1996) *J. Biochem.* 119, 926-933]. The cloned rat CYP51 cDNA was expressed in *Escherichia coli* with modification of the N-terminal amino acid sequence, and the expressed protein (CYP51m) was purified to gel-electrophoretic homogenity. The spectrophotometrically determined specific content of CYP51m was 16 nmol/mg protein and the apparent molecular weight was estimated to be 53,000 on SDS-PAGE. Soret peaks of the oxidized and reduced CO-complex of CYP51m were observed at 417 and 447 nm, respectively. The purified CYP51m catalyzed the 14-demethylation of lanosterol and 24,25-dihydrolanosterol upon reconstitution with NADPH-P450 reductase purified from rat liver microsomes. The apparent K_m and V_{max} values for lanosterol were 10.5 μM and 13.9 nmol/min/nmol P450, respectively, and those for 24,25-dihydrolanosterol were 20.0 μM and 20.0 nmol/min/nmol P450, respectively. The lanosterol demethylase activity of the reconstituted system of CYP51m was inhibited by ketoconazole, itraconazole and fluconazole with apparent IC_{50} values of 0.2, 0.7, and 160 μM, respectively.

Key words: characterization, CYP51, expressed enzyme, P450, sterol 14-demethylase.

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Sterol 14-demethylase P450 (CYP51) is a key enzyme in sterol biosynthesis and is widely distributed in eukaryotes (*1-3*). The final products of sterol biosynthesis differ among animals, plants and fungi in relation to the divergence of the biosynthetic pathways (*4*). As shown in Fig. 1, the 14-demethylation steps are situated after the major divergence point of the pathways and the substrates undergoing 14-demethylation differ among animals, plants and fungi, although the CYP51s are orthologous enzymes derived from an common ancestral protein (*5*). This fact indicates that the substrate specificity of CYP51s has been affected by some structural changes after the divergence of the three major biological kingdoms. Therefore, detailed comparison of the molecular and enzymatic properties of CYP51s from different kingdoms in relation to their structures is an important and interesting subject for evolutionary investigation of CYP51 and sterol biosynthesis.

The molecular and enzymatic properties of yeast CYP51 have been characterized in detail (*6-10*). However, mammalian CYP51 has not yet been characterized so extensively, although it has been purified from rat (*11*) and pig (*12*) liver microsomes. This situation may be due to the difficulty in obtaining a highly purified preparation of mammalian CYP51 with a good recovery because of the low content in liver microsomes.

We have isolated rat and human CYP51 cDNA clones (*13, 14*). Expression of these cDNAs in appropriate host cells is expected to yield highly purified mammalian CYP51 with a good yield. This paper describes the expression of rat CYP51 cDNA in *Escherichia coli*, and the purification and characterization of the expressed protein.

MATERIALS AND METHODS

DNA Preparation and Host Cells—The cDNA of rat CYP51 (pRT-11) was cloned as described previously (*13, 14*). The plasmid vector, pCWori$^+$, and the host, *E. coli* JM109, were the generous gifts from Dr. M.R. Waterman of Vanderbilt University School of Medicine.

Chemicals, Biochemicals, Enzyme Preparations, and Antibodies—Itraconazole and fluconazole, both of which were purified from commercially obtained medicines, were generous gifts from Mr. K. Asai of Takeda Chemical, Osaka. Ketoconazole was obtained from Sigma. Other chemicals, biochemicals and enzymes used in this study were guaranteed reagents obtained from commercial sources. NADPH-P450 reductase was purified from phenobarbital-induced rat liver microsomes according to the method of Yasukochi and Masters (*15*). Anti-rat CYP51 antibodies were raised in a rabbit using a purified preparation of rat CYP51 expressed in *E. coli* and were prepared by the method used for preparing antibodies against yeast

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

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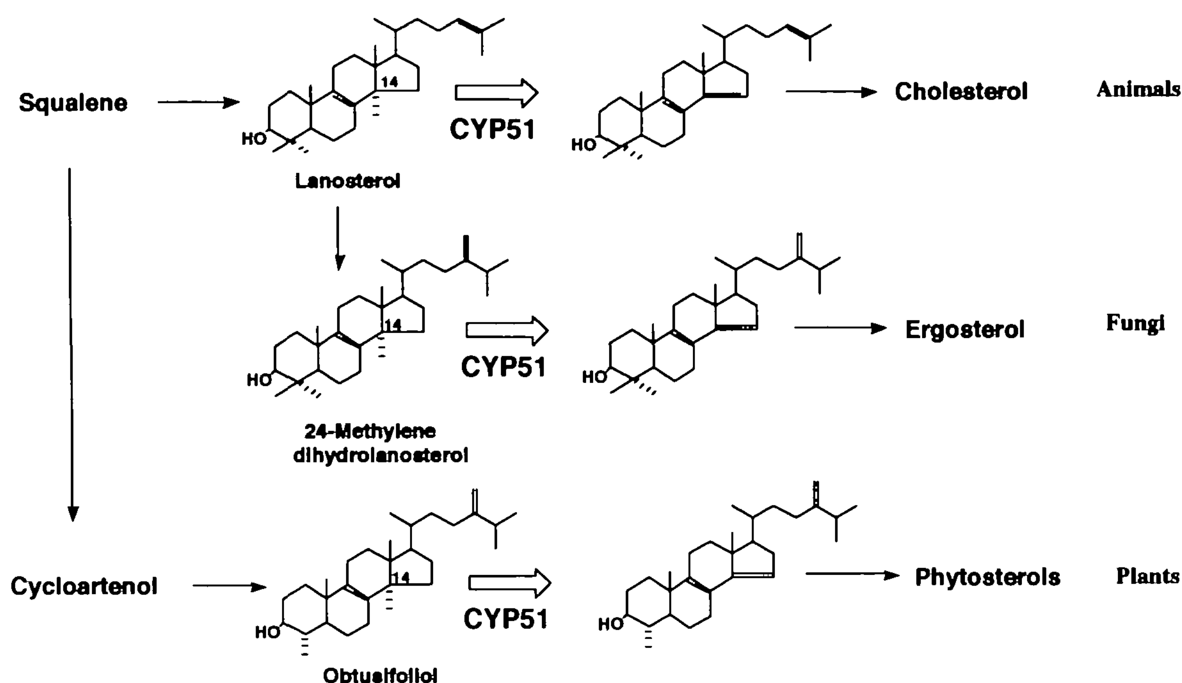

Fig. 1. **The 14-demethylation reactions catalyzed by CYP51 in the sterol biosynthetic pathways of eukaryotes.**

CYP51 (*1*). Restriction endonucleases were purchased from TOYOBO (Tokyo), and the enzymes used for PCR were obtained from Takara Shuzo (Kyoto).

Expression of Rat CYP51 cDNA in E. coli—The strategy for constructing the expression vector for rat CYP51 is shown in Fig. 2. The *Xba*I-*Hin*dIII fragment derived from the pRT-11 plasmid (*13, 14*), which contains the whole sequence of rat CYP51 cDNA (*13, 14*), was inserted into the *Xba*I-*Hin*dIII site of the pCWori$^+$ plasmid to construct the recombinant, pCWratCYP51. To improve the expression efficiency in *E. coli* (*6*), the nucleotide sequence of the pCWratCYP51 plasmid was modified as follows. The DNA cassette covering the initiating ATG to the downstream *Nde*I site of rat CYP51 cDNA having the nucleotide sequence of ATGGCTCTGTTATTAGCAGTTTTT as the initiation site, which was a modification of the corresponding native sequence (ATGGTACTGCTGGGCTTGCTG-CAG), was prepared by PCR. Then, the *Nde*I-*Nde*I region of pCWratCYP51 was replaced with the DNA cassette to construct the expression plasmid, pCWCYP51m. Though this modification, the N-terminal amino acid sequence of rat CYP51 was altered from the native MVLLGLLQ to MALLLAVF. This modification had no apparent effect on the expressed protein (see "RESULTS AND DISCUSSION"), and the modified protein is called "CYP51m" in this paper. The expression plasmids, pCWCYP51m, thus constructed were transformed into *E. coli* JM109 cells by the conventional calcium chloride method. The cells were cultivated overnight at 37°C in Luria-Bertani medium containing ampicillin. An aliquot (3 ml) of this culture was inoculated into 300 ml of Terrific Broth containing ampicillin, followed by incubation at 37°C with constant shaking (250 rpm). When the OD_{550} of the medium reached 0.8, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added to the medium, the temperature of the medium was lowered to 30°C, and the incubation was continued at this temperature under constant shaking. Twenty-four hours after the inoculation, the cells were harvested by centrifugation and used as the CYP51m-expressing cells.

Preparation of a Membrane Fraction of E. coli—CYP-51m-expressing *E. coli* cells were suspended in 0.1 M potassium phosphate buffer, pH 7.5, and then disrupted twice with a French press at an output pressure of 1,200 kg/cm^3. The cell-free homogenate thus obtained was centrifuged at 3,000$\times g$ for 10 min. The supernatant obtained on this centrifugation was further centrifuged at 146,000$\times g$ for 2.5 h, and the precipitate obtained on this centrifugation was suspended in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 20% glycerol, and then stored anaerobically at -80°C as the membrane fraction of *E. coli*.

Purification of CYP51m Expressed in E. coli—All manipulations were carried out at 2-4°C. The membrane fraction of *E. coli* (400 mg protein) was suspended in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 1 mM DTT, 20% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride to give a protein concentration of 5 mg/ml. Sodium cholate was added to the suspension to the concentration of 1.0% and then the suspension was stirred for 1 h. The suspension was centrifuged at 146,000$\times g$ for 2.5 h, and then the supernatant containing the solubilized CYP51m was dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.5 mM DTT, 0.3% sodium cholate, and 20% glycerol. After removing insoluble substances by centrifugation at 146,000$\times g$ for 60 min, the concentration of sodium cholate in the dialyzed solution was adjusted to 0.5%. The solution was then applied to a column (1.5$\times$9.5 cm) of EAH Sepharose 4B equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.5% sodium cholate, and 20% glycerol. The column was washed with the equilibration buffer, and then the adsorbed CYP51m was eluted with

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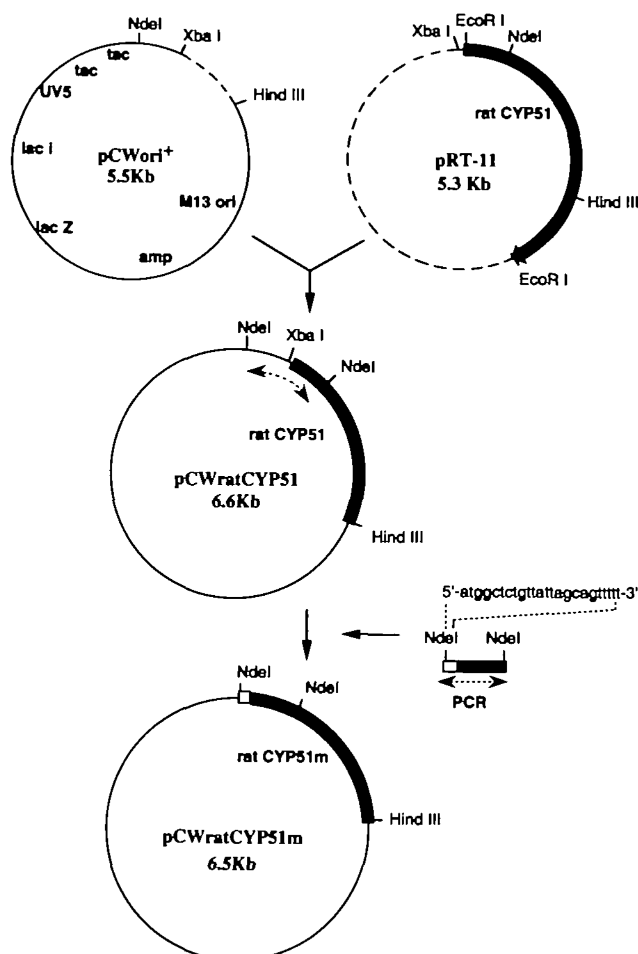

Fig. 2. **Strategy for constructing the expression vector, pCW**CYP51m.

10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.3% sodium cholate, 0.1% Emulgen 913, and 20% glycerol. The CYP51m in the eluate was further purified by successive chromatographies on DE52 and Bio-Gel HT columns according to the previously described method developed for purifying yeast CYP51 (*6*).

Analytical Methods—The P450 content was determined spectrophotometrically. To determine P450 in the membrane fraction and crude preparations, the extinction coefficient difference of the reduced CO-difference spectrum (91.1 $mM^{-1}\cdot cm^{-1}$) (*17*) was used. The P450 content of the purified preparation was determined from the absorbance of the Soret peak (417 nm) using the extinction coefficient of 101 $mM^{-1}\cdot cm^{-1}$ determined for yeast CYP51 (*6*). Protein was determined by the method of Lowry *et al.* (*18*) using bovine serum albumin as the standard.

Assaying of the Enzymatic Activity—The enzymatic activity of the purified CYP51m was assayed with the reconstituted system consisting of CYP51m and purified rat NADPH-P450 reductase. The reaction mixture (1.0 ml) comprised 0.011 nmol CYP51m, 0.2 unit NADPH-P450 reductase, 20 nmol substrate (lanosterol or 24,25-dihydrolanosterol dispersed with 0.05 mg of dilauroylphosphatidyl choline), 50 mM potassium phosphate buffer, pH 7.5, 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 conversion ratio of the substrate to the 14-demethylated product by the previously described method (*1*). To determine the inhibitory effects of ketoconazole, itraconazole and fluconazole on CYP51m, these compounds dissolved in dimethylsulfoxide were added to the above reaction mixture.

Determination of the Binding of Azole Compounds to CYP51m—The binding of azole compounds to CYP51m was determined by measuring the azole-induced spectral change of the oxidized CYP51m, and the affinity of azole compounds to CYP51m was determined by spectrophotometric titration.

RESULTS AND DISCUSSION

Expression of CYP51m in E. coli—*E. coli* JM109 cells transformed with the expression plasmid, pCWCYP51m, produced P450 upon cultivation under the conditions described under "MATERIALS AND METHODS." Most of the expressed P450 was recovered in the membrane fraction of the cells. The P450 content of the membrane fraction was determined to be 0.27 nmol/mg protein and the yield of the expressed P450 recovered in the membrane fraction was calculated to be 131.4 nmol/liter of culture. This yield was high enough for use of this expression system as the source for purifying rat CYP51. The modification of the nucleotide sequence covering the translation initiation point into the AT-rich sequence (see "MATERIALS AND METHODS"), which was developed by Dr. Waterman's group for the expression of mammalian P450s in the same host-vector system (*16, 19*), was effective for the expression of rat CYP51.

Upon aerobic incubation with NADPH-P450 reductase purified from rat liver microsomes and NADPH, the membrane fraction of *E. coli* containing CYP51m catalyzed the 14-demethylation of lanosterol with an activity level of 1.45 nmol/min/nmol P450. This indicated that CYP51m expressed in *E. coli* membranes retained the catalytic activity of CYP51 in spite of the substitution of its N-terminal 8 amino acids. Then, we purified CYP51m from this membrane fraction to obtain a purified preparation of rat CYP51.

Purification of CYP51m from the Membrane Fraction—The CYP51m expressed in the membrane fraction was solubilized with 1% sodium cholate. The solubilized CYP51m was purified by the method described under "MATERIALS AND METHODS." The behavior of CYP51m in the chromatographic steps for the purification, *i.e.* on EAH Sepharose 4B, DE52 and Bio-Gel HT, was essentially the same as that of yeast CYP51 (*6*). This suggested that the properties of the molecular surface of CYP51m were substantially similar to those of yeast CYP51 although the amino acid sequence identity between them was only about 40% (*13*).

The specific content of the purified preparation was 16.0 nmol/mg protein and the yield from the membrane fraction was nearly 30%. Figure 3 shows the results of SDS-PAGE and Western blot analyses of CYP51m at each purification step. As shown in the right panel of Fig. 3, three protein bands positive for anti-rat CYP51 antibodies were observed at M_r values of 45,000, 52,000, and 53,000 for the

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crude preparations (lanes 1 through 3). Two small bands disappeared on the successive column chromatography, and the final preparation (lane 6) was electrophoretically (left panel) and immunochemically (right panel) homogenous, with an apparent M_r of 53,000. These results seem to suggest the possibility that the two smaller bands (M_r = 45,000 and M_r = 52,000) represent shorter products of the transformed CYP51m, which could be removed by the chromatographic steps. Since P450 eluted from the columns was monitored as to the absorption at 417 nm, these smaller products might be colorless incomplete P450 or very unstable proteins decomposed during purification. The apparent M_r value of the expressed CYP51m (53,000) was somewhat smaller than the theoretical molecular weight (56,700) calculated from the amino acid sequence. However, such a discrepancy between an apparent M_r value determined on SDS-PAGE and a theoretical molecular weight for the same protein has usually been observed for other P450s. Furthermore, the same apparent M_r value of 53,000 was obtained for CYP51 in rat liver microsomes on Western blotting (data not shown). Accordingly, the purified preparation obtained here is considered to be the complete CYP51m molecule.

Spectrophotometric Properties—Figure 4 presents the absorption spectra of the purified CYP51m. These spectra were characteristic of low-spin P450. The Soret peaks of the oxidized form and reduced CO complex were observed at 417 and 447 nm, respectively. Similar absorption spectra were reported by Trzaskos *et al.* (*11*) for rat CYP51, and by Sono *et al.* (*12*) for pig CYP51. However, the absorption spectra reported by them were apparently different from those of yeast CYP51, especially in the unusually low Soret peaks of the reduced CO-complexes. As shown in Fig. 4, the Soret peak of the reduced CO-complex of CYP51m showed normal intensity and the absorption spectra of CYP51m were superimposable on those of yeast CYP51. Thus, it is concluded that the spectral properties of rat and yeast CYP51s are essentially the same, suggesting similar heme environments of rat and yeast CYP51s. The absorption peak of the reduced CO complex of CYP51m gradually changed from 447 to 420 nm, indicating its slow denaturation to a P420 form. Such a phenomenon was not observed for yeast CYP51. Therefore, the stability of the reduced CO complex of CYP51m might be lower than that of yeast CYP51. Aoyama *et al.* (*20*) reported that the reduced CO-complex of *Mycobacterium tuberculosis* CYP51 expressed in *E. coli* was rapidly denatured to a P420 form. These facts suggest that the stability of the reduced CO-complex of CYP51 significantly differs with the origin.

Catalytic Properties—The reconstituted system consisting of the purified CYP51m and NADPH-P450 reductase purified from rat liver converted lanosterol and 24,25-dihydrolanosterol to the corresponding 14-demethylated and 14,15-unsaturated products, 4,4-dimethylcholesta-8,14,24-trienol, and 4,4-dimethylcholesta-8,14-dienol (Fig. 5), respectively, indicating that the purified CYP51m has the property of sterol 14-demethylase. The kinetic parameters of sterol 14-demethylation by CYP51m are summarized in Table I. The K_m value of CYP51m for 24,25-dihydrolanosterol was nearly twice that for lanosterol. However, the V_{max} values of CYP51m were also higher for 24,25-dihydrolanosterol than for lanosterol, and V_{max}/K_m values of CYP51m for these compounds were nearly comparable. Therefore, it is concluded that CYP51m metabolizes both of these compounds with similar efficiency, although it shows higher affinity for lanosterol than for 24,25-dihydrolanosterol. Rat CYP51 has already been purified by Trzaskos *et al.* (*11*), but the K_m and V_{max} values were not determined for this preparation. The K_m values of purified pig CYP51 (*12*) for lanosterol and 24,25-dihydrolanosterol were reported to be 8.7 and 22.7 μM, respective-

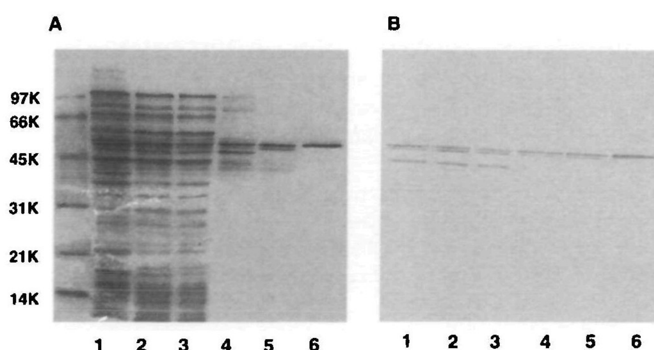

Fig. 3. **SDS-PAGE and Western blot analysis of protein specimens isolated at each step of purification of CYP51m.** A: SDS-PAGE of protein specimens from each purification step. The amounts of protein applied to the gel were 10 μg for lane 1, 5 μg for lanes 2 and 3, and 0.5 μg for lanes 4, 5, and 6. The gel comprised 10% polyacrylamide cross-linked with 0.1% *N,N'*-methylenebisacrylamide, and proteins were stained with Coomassie Brilliant Blue R250. Molecular mass standards were phosphorylase B (97K), bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21K), and lysozyme (14K). B: Western blot analysis of the protein bands separated by SDS-PAGE. The amounts of protein applied to lanes 1-3 and lanes 4-6 were 0.5 and 0.05 μg, respectively. Immunostaining was performed with anti-rat CYP51 IgG and the alkaline phosphatase-conjugated goat IgG against rabbit IgG as the primary and secondary antibodies, and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as color reagents. Lanes 1, 2, 3, 4, 5, and 6 correspond to the membrane fraction of *E. coli*, the cholate-solubilized fraction, the eluate from the EAH Sepharose 4B column, the pass-through fraction of the DE 52 column, and the eluate from the Bio-Gel HT column, respectively.

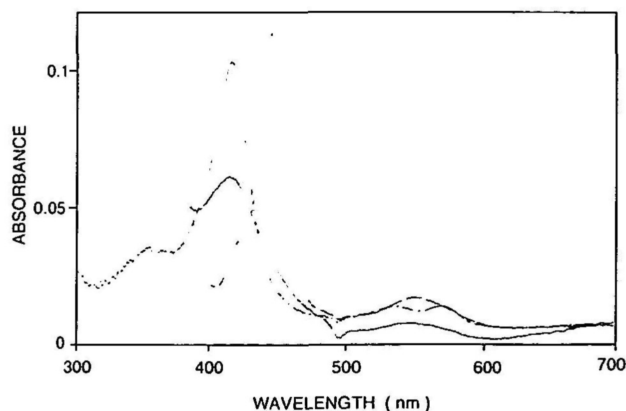

Fig. 4. **Absorption spectra of purified CYP51m.** Purified CYP51m (1.10 μM) was dissolved in 100 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol and 0.2% Emulgen913. Broken line with points: Oxidized form. Solid line: Form reduced with sodium dithionite. Broken line: CO complex of the reduced form.

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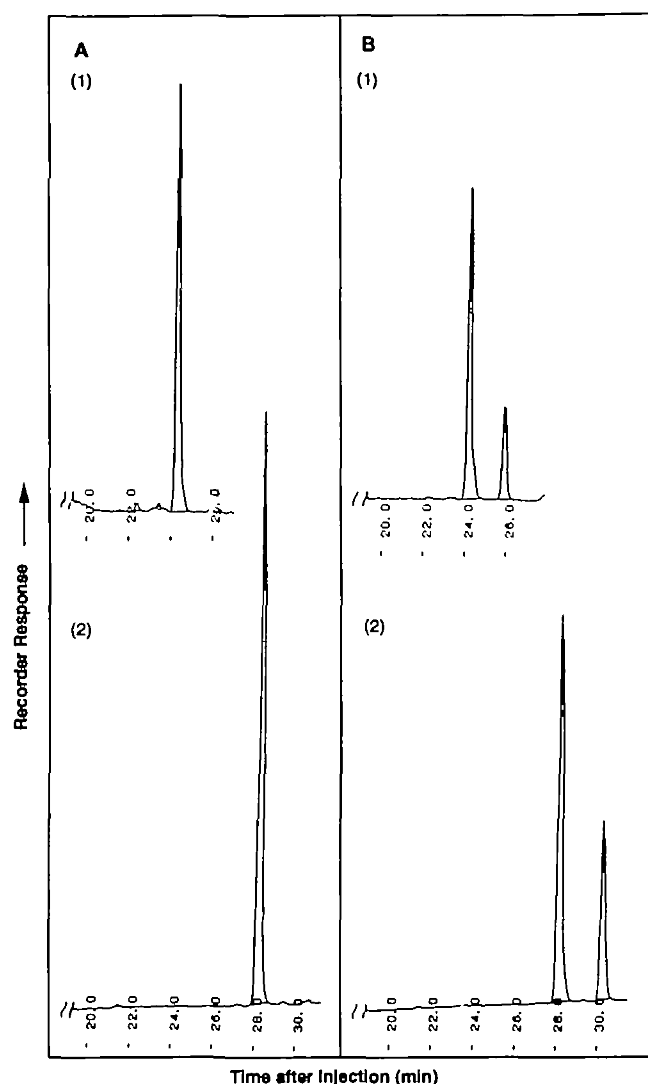

Fig. 5. **Gas-chromatograms of sterols in the reaction mixture of the 14-demethylase assay with the CYP51m reconstituted system.** The 14-demethylase reaction with CYP51m was carried out as described under "MATERIALS AND METHODS." Sterols extracted from the reaction mixture were trimethylsilylated and then subjected to gas-liquid chromatography on a DB-17 fused-silica capillary column at 225°C. Panel A, chromatograms of the trimethylsiyl sterols extracted from the reaction mixture containing 24,25-dihydrolanosterol (1) or lanosterol (2) as the substrate at time zero of the incubation. Panel B, chromatograms of the trimethylsilyl sterols extracted from the same reaction mixtures after 10 min of the enzyme reaction.

ly, and these values were comparable to those of CYP51m. However, the V_{max} values (1.02 and 0.63 nmol/min/nmol P450 for lanosterol and 24,25-dihydrolanosterol, respectively) were less than one-tenth of those obtained for CYP51m (Table I). Since the V_{max} values obtained with a reconstituted P450 system depended on the reconstitution conditions, the low V_{max} values of the pig CYP51 system (*12*) might be due to incompleteness of the reconstitution conditions. The V_{max} value of yeast CYP51 for lanosterol obtained with the reconstituted system (*1*) was usually in the range of 10 to 20 nmol/min/nmol P450. It can thus be concluded that the K_m and V_{max} values of CYP51m for

TABLE I. **Kinetic parameters of 14-demethylase activity of CYP51m in the reconstituted system with NADPH-P450 reductase.** Sterol 14-demethylase activity was determined as described under "MATERIALS AND METHODS" with various concentrations of substrates.

Substrate	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m
Lanosterol	10.5	13.9	1.3
Dihydrolanosterol	20.0	20.0	1.0

TABLE II. **Inhibitory effects and binding of typical azole antifungal compounds to CYP51m.** IC_{50} values were determined from the inhibition of lanosterol 14-demethylase activity by CYP51m assayed under the conditions described under "MATERIALS AND METHODS." K_d values were determined by means of the spectrophotometric titration described in Fig. 6.

Compound	IC_{50} (μM)	K_d (μM)
Ketoconazole	0.18	<0.4
Itraconazole	0.33	0.78
Fluconazole	170	160

lanosterol and 24,25-dihydrolanosterol shown in Table I are reasonable ones for CYP51. Using rat liver microsomes as the enzyme preparation, we found that rat CYP51 favorably utilized lanosterol as the substrate at a low substrate concentration, however, 24,25-dihydrolanosterol could be metabolized as well as lanosterol when the substrate concentration was increased to 20 μM (*21*). The kinetic parameters of CYP51m shown in Table I agreed with the above-mentioned substrate selectivity of rat liver microsomal CYP51. Thus, we concluded that the kinetic parameters of CYP51m might represent the catalytic properties of rat CYP51. In the previous paper (*21*), we described that the substrate specificity of rat liver microsomal CYP51 was different from that of yeast CYP51, which did not metabolize 24,25-dihydrolanosterol as favorably as lanosterol, and the substrate selectivity of CYP51m described here confirms this conclusion. Since 24,25-dihydrolanosterol is the sterol intermediate existing only in the cholesterol biosynthetic pathway in animals, the above-mentioned difference in the substrate specificity of rat and yeast CYP51s must be responsible for their metabolic roles, as discussed in the previous paper (*5*).

Inhibitory Effects of Azole Antifungal Agents—The lanosterol 14-demethylase activity of CYP51m was inhibited by azole antifungal agents, such as fluconazole, ketoconazole and itraconazole (Table II). Figure 6A presents a typical spectral change induced by ketoconazole, and the other two compounds induced essentially the same spectral changes (data not shown). The spectral change shown in Fig. 6A was superimposable on that of yeast CYP51 (*22*), indicating that CYP51m interacts with azole antifungal agents in the same manner as yeast CYP51 does. By means of spectrophotometric titration using the absorbance difference between 433 and 417 nm in the different spectra (upper spectrum in Fig. 6A), the binding of ketoconazole to CYP51m was determined (Fig. 6B). The titration curve (Fig. 6B) indicated that CYP51m formed a one to one complex with ketoconazole, and the same results were obtained with fluconazole and itraconazole (data not shown). The apparent K_d values of ketoconazole, itracon-

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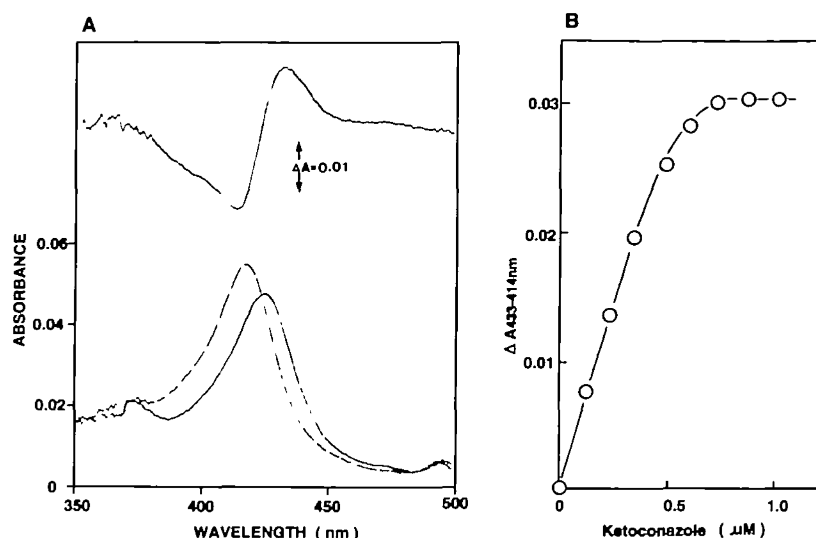

Fig. 6. **Ketoconazole-induced spectral change and spectrophotometric titration of CYP51m with ketoconazole.** A: Absorption spectra of oxidized CYP51m (broken line) and its ketoconazole complex (solid line). The concentrations of CYP51m and ketoconazole were 0.5 and 0.8 μM, respectively. Upper spectrum, ketoconazole-induced difference spectrum of oxidized CYP51m. B: Oxidized CYP51m (0.5 μM) was titrated with the indicated concentrations of ketoconazole, and the magnitudes of the resulting difference spectra (absorbance difference between 433 and 414 nm) were plotted as a function of the ketoconazole concentration.

TABLE III. **Comparison of inhibitory effects of azole antifungal agents on CYP51m and *S. cerevisiae* CYP51.** Lanosterol 14-demethylase activity of CYP51m was determined as described under "MATERIALS AND METHODS" in the presence and absence of the indicated azole compounds. The same activity of CYP51 of *S. cerevisiae* was assayed by the previously described method (*1*) in the presence of the indicated azole compounds. The CYP51 concentration was 0.013 μM in both experiments.

Compound	CYP51m		*S. cerevisiae* CYP51	
	Concentration	Inhibition	Concentration	Inhibition
Ketoconazole	0.2 μM	45%	0.02 μM	100%
Itraconazole	0.2 μM	10%	0.02 μM	100%
Fluconazole	0.2 μM	0%	0.02 μM	75%

azole and fluconazole determined on the spectral titration are summarized in Table II. The K_d values obtained for ketoconazole and itraconazole were higher than their IC_{50} values (Table II). However, the K_d values of ketoconazole and itraconazole might have been overestimated, because the P450 concentration necessary for the spectral titration (0.5 μM) was higher than their IC_{50} values, and the titration curves of these compounds were linear in the low concentration range, as shown in Fig. 6B. Accordingly, the true K_d values of these compounds for CYP51m may be comparable to their IC_{50} values. It can thus be concluded that the inhibition of CYP51m by an azole compound is caused by the formation of a one to one complex with an azole compound. It is noteworthy that the sensitivity of CYP51m to these azole compounds was considerably lower than that of yeast CYP51 (Table III). These compounds inhibited yeast CYP51 almost completely at 0.02 μM, which was a comparable concentration to that of CYP51 (0.013 μM) in the reaction mixture (Table III). In contrast, lanosterol 14-demethylation by CYP51m was not completely inhibited even in the presence of 0.2 μM of the azoles (Table III), which was a 20 times higher concentration than that of CYP51m in the reaction mixture. The low susceptibility of CYP51m to these azoles is also evident from the IC_{50} values shown in Table II. The low sensitivity of rat liver microsomal CYP51 to azole compounds has been reported also for isoprenoid-containing azole compounds (*23, 24*). Accordingly, a relatively low sensitivity to azole compounds, which act as potent inhibitors for yeast CYP51, is another conspicuous feature of rat CYP51.

General Discussion—Taking all the findings obtained with the expressed CYP51m together, it is evident that the basic molecular characteristics of rat CYP51 are closely similar to those of yeast CYP51, as expected from their orthologous relationship. However, the substrate selectivity and sensitivity to azole inhibition of CYP51m were clearly different from those of yeast CYP51. Since CYP51m is considered to retain the properties of the native rat CYP51, as discussed in the previous sections, these differences observed between CYP51m and yeast CYP51 must reflect the difference between rat and yeast CYP51s. Substrate selectivity must be determined by the local structure of the substrate-recognition sites, and azole antifungal agents interact with the substrate-binding site or in the vicinity of CYP51 (*25*), and rat and yeast CYP51s are orthologous enzymes derived from a common ancestor (*5*). Accordingly, these differences are considered to be due to amino acid substitutions occurring in the active sites of the enzyme after the divergence of animals and fungi. Such differentiation might have played an important role in the establishment of the different sterol biosynthetic pathways in animals and fungi, because the difference observed between rat and yeast CYP51s coincides well with the difference in intermediates undergoing 14-demethylation. Actually, some characteristic amino acid substitutions were found around the putative substrate-recognition sites of mammalian and fungal CYP51s (*5*). By using the expression system of CYP51m established in this study and site-directed mutagenesis, we are analyzing the essential amino acid residues responsible for the different substrates and azole selectivities observed for mammalian and fungal CYP51s.

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