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The G464S Amino Acid Substitution in *Candida albicans* Sterol 14α-Demethylase Causes Fluconazole Resistance in the Clinic through Reduced Affinity

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Fluconazole selectively inhibits fungal sterol 14α-demethylase, a cytochrome P450 enzyme found in plants, animals, fungi, and *Mycobacteria*. The mutation G464S, observed in the heme-binding domain of sterol 14α-demethylase in clinical strains of fluconazole-resistant *Candida albicans*, is shown here to cause resistance through substantially reducing the inhibitory effect of fluconazole and is associated with perturbation of the heme environment as indicated by spectral data. The protein exhibits 42% of the maximal enzymatic rate of the wild-type protein allowing continued production of the end product of fungal sterol biosynthesis, ergosterol, in resistant strains. This mutation may cause these phenotypes through altering the heme location, thus changing the ability of residues above the heme to bind the drug effectively. This perturbation would also account for the observation of reduced sterol demethylase catalytic activity by changing the location of the 14α-methyl group in relation to oxygen-bound heme during the catalytic cycle.

The introduction of orally active antifungal azole drugs since the 1980s, particularly fluconazole, was a significant development allowing treatment of systemic fungal infections without the problem of nephrotoxicity associated with amphotericin B treatment. This was valuable as since this time the incidence of fungal infection has increased dramatically, mainly associated with the immuno-compromised host suffering AIDS, after organ transplantation and chemotherapy as well as in intensive care [1]. Of the fungal infections encountered candidiasis involving *Candida albicans* is most common, although other fungi normally associated with being safe, e.g., *Saccharomyces cerevisiae* and some associated with plant disease, e.g., *Fusarium* are now observed to cause infection. Due to the prolonged period of therapy, coupled with a fungistatic activity, resistance to the main antifungal used, fluconazole, has been increasing and is life-threatening. Reports indicate the frequency in late-stage AIDS patients exceeds 10% of patients [2].

The target of the azole drugs is sterol 14α-demethylase (Erg11p; cytochrome P45051) which participates in ergosterol biosynthesis [3]. Inhibition results in a build up of substrate, which is metabolized by *Candida albicans* to give rise finally to 14α-methylergosta-8,24(28)-dien-3β,6α-diol (Fig. 1). Formation of the diol has been indicated to be central to the mode of action of azoles and sterol C5-desaturase is needed for the 6-hydroxylation [4]. Resistance can occur through defective sterol C5-desaturation [5] and recent work has also shown lanosterol, substrate of CYP51, can support yeast growth [6]. The lethality of mutants of yeast defective in CYP51, which also accumulate diol, can be suppressed by sterol C5-desaturase, such as in the first defective missense mutant of *S. cerevisiae CYP51 (ERG11)* studied, SG1 [7, 8]. The resistance of this strain to azole antifungal can be ascribed to the associated defect in sterol C5-desaturation rather than the CYP51 defect [8]. Recently, we observed polymorphisms of *ERG11* in fluconazole resistant clinical strains of *Candida albicans* [9], without associated cellular defects in sterol C5-desaturase or ergosterol biosynthesis. One of the changes observed most frequently, corresponding to the substitution G464S, has been subsequently reported in further clinical strains and associated with resistance in genetic studies [10, 11]. We show here that the mutant protein exhibits reduced fluconazole affinity and catalytic activity in sterol 14α-demethylation and this can account for the resistant phenotype ob-

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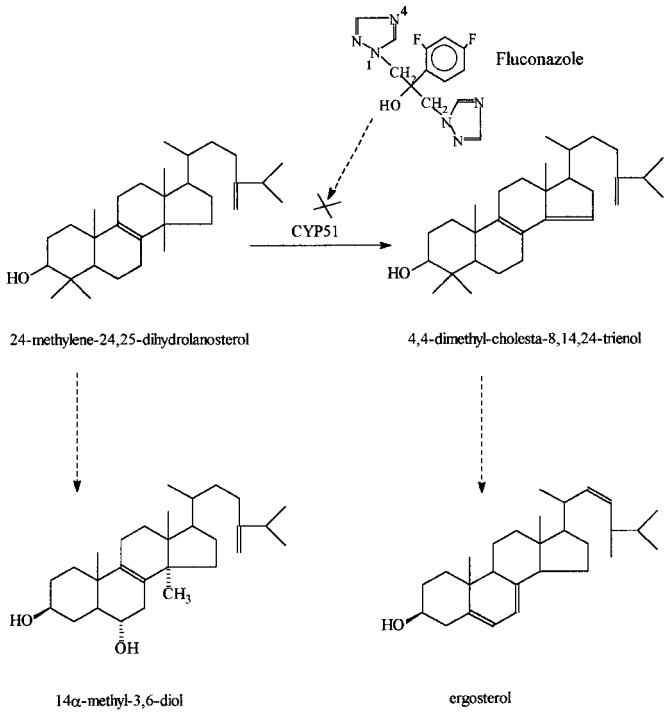

FIG. 1. Sterol 14α-demethylation catalyzed by *C. albicans* CYP51 during ergosterol biosynthesis. Fluconazole inhibits this biosynthetic step through coordination of the nitrogen atom in the azole ring to the heme iron of the enzyme with the N1 substituent groups interacting with the apoprotein. Inhibition results in a build up of substrate which is metabolized by *C. albicans* to give rise finally to 14α-methylergosta-8,24(28)-dien-3β,6α-diol, which ultimately results in cell growth arrest.

served. No previous reports have proved amino acid substitution causes fluconazole resistance in the clinic through altered drug affinity of the CYP51 target.

MATERIALS AND METHODS

Site-directed mutagenesis. Our previous studies have employed a yeast expression system to express *Candida albicans CYP51* using the *Saccharomyces cerevisiae* GAL 10 promoter in the vector YEp51 [12]. Recombinant PCR was employed to replace the codon 464 encoding glycine (GGT) with one encoding serine (AGT). The following oligonucleotides were used as outside primers: 1, 5′-AAATTTGCTAAAGCTGCTTTGACTACT-3′ annealing to position 429–456 of *C. albicans CYP51* and 2, 5′-ACTAACGTTTTA-AAACATACAAGTTTCTCT-3′ annealing to the 3′ end at position 1569–1587 of the gene. Inside primers used in the PCR mutagenesis were: 1, 5′-TATTTACCATTT**AGT**GGTGGTAGACATAG-3′ and 2, 5′-CTATGTCTACCACC**ACT**AAATGGTAAATA-3′. In a first step, two separate PCRs were carried out using outside primer 1/inside primer 2 and inside primer 1/outside primer 2, respectively. The partially overlapping DNA fragments obtained were purified, mixed and recombined in a subsequent PCR step using outside primer 1/outside primer 2. PCRs were performed on a Perkin–Elmer DNA thermal cycler with conditions as previously described [12]. PCR was undertaken using Pfu polymerase (Stratagene). The resulting DNA fragment containing the G464S mutation was digested with *Nsi*I/*Hin*dIII and ligated into the YEp51:CYP51 expression plasmid according to Fig. 1. Introduction of the mutation and maintenance of the authentic sequence was corroborated by DNA sequencing. All restriction enzymes and T4DNA ligase were obtained from Promega and the recommended conditions for use were applied.

Heterologous expression of recombinant proteins. The plasmid YEp51:CYP51(G464S) was transformed into *S. cerevisiae* strain AH22 (MATa *leu2-3,2-112 his3-11,3-15 can*^{r}). Yeast transformants were grown at 28°C, 250 rpm with 250 ml culture in 500-ml flasks. The media used consisted of Difco yeast nitrogen base without amino acids [1.34% (w/v)] supplemented with 100 mg/liter histidine and 2% (w/v) glucose as initial carbon source. Heterologous expression was induced when the glucose was exhausted at a cell density of approximately 10^8 cells/ml. The culture was left a further four hours before galactose was added to a concentration of 3% (w/v). After 20 hours induction cells were harvested by centrifugation, resuspended in buffer containing 0.4 M sorbitol, 50 mM Tris–HCl, pH 7.4 and broken using a Braun disintegrator (Braun GmbH, Mesungen, Germany) with four bursts of 30 s together with cooling from liquid carbon monoxide. Cell debris was removed by centrifugation at 1500*g* for 10 min using a MSE bench centrifuge. The resulting supernatant was centrifuged twice at 10,000*g* for 20 min to remove mitochondria and then at 100,000*g* for 90 min to yield the microsomal pellet. This was resuspended using a Potter–Elvehjeim glass homogenizer at about 10 mg protein/ml in the same buffer described above. P450 concentration by reduced carbon monoxide difference spectroscopy was measured as described previously [13] and protein concentrations accordingly [14].

Spectrophotometric studies with recombinant proteins. Interaction of fluconazole with *C. albicans* wild type CYP51 and CYP51(G464S), isolated following heterologous expression in *S. cerevisiae,* was analyzed spectrophotometrically by using a Hitachi-U3010 recording spectrophotometer. Microsomal wild type CYP51 and CYP51(G464S), 0.2 nmol in 0.1 M potassium phosphate buffer, pH 7.2, containing 20% glycerol, was titrated with fluconazole. Further details are given in the figure legends.

Determination of microsomal sterol 14α-demethylase activity and inhibition by fluconazole. Our previous studies have developed a novel method for studying sterol 14α-demethylase activity of recombinant CYP51 following heterologous expression in yeast [12]. Following the addition of the 32-tritiated CYP51 substrate [32-^{3}H]-3β-hydroxylanost-7-en-32-ol, (52 μg in 10 μl of dimethylformamide) to the microsomal protein, NADPH (final concentration 1 mM) was added to the reaction mixture and incubated at 37°C. After 20 min, the reaction was stopped by the addition of a mixture of dichloromethane (2 ml) and water (2 ml) and immediately shaken and then centrifuged. The organic layer was discarded and further dichloromethane (2 × 2 ml) was added and the above procedure repeated. To the resulting aqueous phase was added charcoal, the suspension shaken, left at 4°C for 1 h and finally centrifuged to remove the charcoal. The radioactivity of the aqueous phase was measured by liquid scintillation counting. Sterol 14α-demethylase activity of the microsomal preparations of wild type CYP51 and CYP51(G464S) was assayed as described above. Fluconazole was added to the reaction mixtures respectively from 1000-fold stock solutions.

RESULTS

Site-directed mutagenesis and heterologous expression of CYP51 and CYP51(G464S). The expression system, which we have previously developed, had already included modifying the coding sequence at 263 due to the presence of CTG which encodes leucine in *S. cerevisiae,* but serine in *C. albicans* [15]. Introduction of TCT allowed the authentic amino acid (serine) to be included when expressed in *S. cerevisiae.* Further mutagenesis was undertaken to change Gly464 to Ser

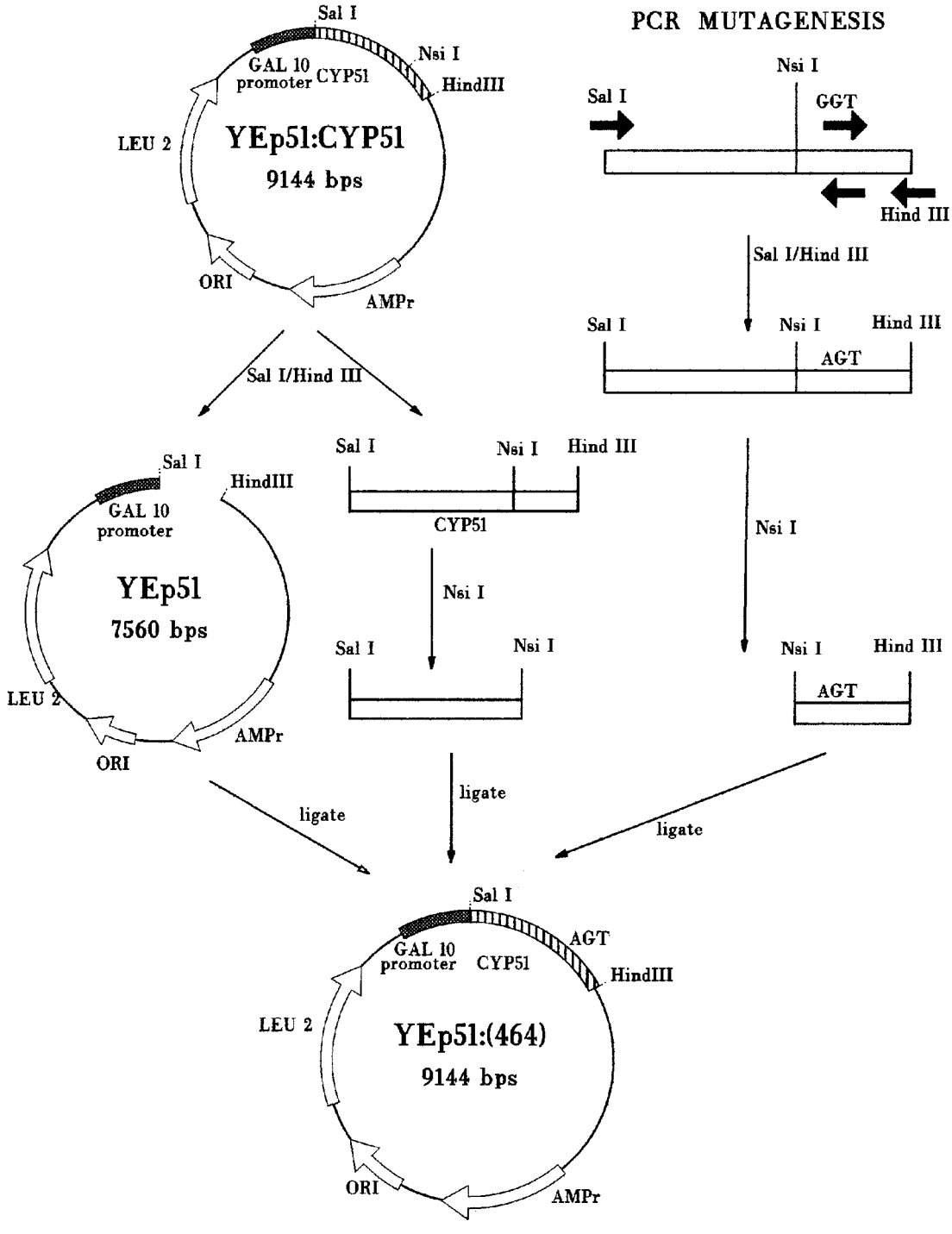

FIG. 2. Schematic representation of the strategy used for the generation of CYP51(G464S). Site-directed PCR mutagenesis was performed to change the triplet at 464 from GGT to AGT. The *Nsi*I–*Hin*dIII mutant fragment was ligated into the corresponding region of the wild-type gene in the yeast expression vector YEp51 allowing expression from the GAL10 promoter.

involving a single base substitution of GGT>AGT which is outlined in Fig. 2. *S. cerevisiae* strain AH22 transformed with CYP51(G464S) expressed comparable levels of P450 to wild-type enzyme with up to 2.5 nmol P450/mg microsomal protein produced after expression from the GAL10 promoter of YEp51 when determined by the optical absorption spectrum of the carbon monoxide-bound form of reduced P450. The absorption maximum of the CO-bound form of wild-type CYP51 was located at 447 nm. However, the CO-bound form of CYP51(G464S) had a Soret absorption maximum at 450 nm, which reflects

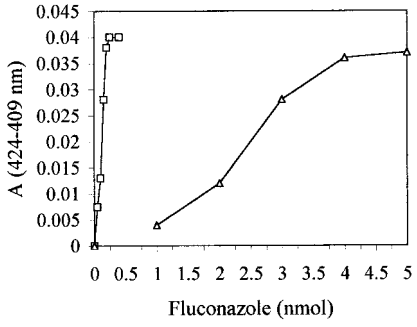

FIG. 3. Comparative analysis of Type II binding spectra. The magnitude of spectra obtained with different amounts of fluconazole are shown bound to 0.2 nmol CYP51 (□) and CYP51(G464S) (△).

slight perturbation in the heme environment of the protein.

Binding of fluconazole to CYP51 and CYP51(G464S). Fluconazole caused characteristic changes in the oxidized forms of both CYP51 and CYP51(G464S) due to a shift to low spin state for the heme on binding fluconazole. This interaction gave rise to the Type II change which is characterized by the displacement of the native sixth ligand of the heme iron (water molecule) by the nitrogen atom in the triazole ring (*N4*) of fluconazole. This shift resulted in a spectral peak (420–427 nm) and a corresponding trough (390–410 nm) [16]. The intensity of the resulting difference spectrum was found to be proportional to the amount of the azole bound form of the cytochrome [17]. Fluconazole produced the Type IIa spectral change on binding to both CYP51 and CYP51(G464S). The spectral peak was located at 424 nm and the trough located at 409 nm for wild-type CYP51 and CYP51(G464S). The apparent affinity of fluconazole for CYP51(G464S) was greatly reduced when compared to the wild-type CYP51. Fluconazole bound stoichiometrically with wild-type CYP51 whereas the compound only formed one to one complexes with CYP51(G464S) at approximately 15-fold excess amount of fluconazole over P450 (Fig. 3).

Sterol 14α-demethylase activity of CYP51 and CYP51(G464S) and inhibition by fluconazole. The catalytic activities of CYP51 and CYP51(G464S) were assayed by measuring the release of [^{3}H]-formic acid during the conversion of [32-^{3}H]-3β-hydroxylanost-7-en-32-ol to its C14 demethylated product, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol. Heterologously expressed microsomal CYP51(G464S) showed a reduction in its ability to metabolize the alcohol derivative substrate compared to the wild-type P45051 (58% reduction in the catalytic activity of CYP51(G464S) compared to CYP51). As expected, the demethylation activity was below the baseline of detectability in microsomes from the host strain harboring the empty parent vector YEp51 (data not shown). Wild type CYP51 and CYP51(G464S) were shown to have similar K_m values (20 μM). However, the maximal enzymatic rate (V_{max}) differed for both proteins (240 pmol product formed/minute/nmol CYP51 compared to 100 pmol product formed/minute/nmol CYP51(G464S)) showing reduced catalytic activity of the mutant enzyme compared to the wild type form.

Figure 4 shows the results of experiments in which CYP51 and CYP51(G464S) activity was inhibited with increasing amounts of fluconazole. For CYP51, the inhibition of enzymatic activity was dependent on azole amount, and total inhibition of enzyme activity occurred at an equimolar amount of fluconazole to that of CYP51 (IC_{50} = 1.5 nmol). However, for CYP51(G464S) (IC_{50} = 13.75 nmol), a 9-fold excess level of fluconazole was required to inhibit sterol 14α-demethylase activity by 50%.

DISCUSSION

The emergence of resistance to azole antifungals has become a common and life-threatening clinical problem in candidiasis associated with late-stage AIDS patients and in patients undergoing chemotherapy, after organ transplantation as well as in intensive care [18, 19]. Fluconazole has been very successful in treating candidiasis with high bioavailability and tissue penetration, but like all azole drugs it exerts a fungistatic, not a fungicidal effect, and requires in AIDS patients lifetime and sometimes prophylactic treatment. The prolonged period of treatment, perhaps coupled to the fungistatic action in which viable cells may remain, led to a situation in which resistant strains were selected.

As azole antifungals are the only compounds in advanced clinical trial understanding, and diagnosing, the mechanisms of resistance is important for devising the therapeutic response to resistance which may arise by more than one mechanism. It was apparent from our detection of amino acid polymorphism in the target

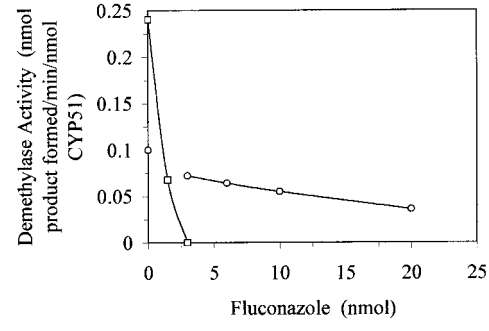

FIG. 4. Inhibition of sterol 14α-demethylase activity. Comparative plot of sterol demethylase activity in the presence of varying amounts of fluconazole for CYP51 and CYP51(G464S). P450 was used to examine the inhibitory effects of fluconazole for CYP51 (□) and CYP51(G464S) (○).

Human	CYP51	F **G** A G R H R C I G E N F
Rat	CYP51	F **G** A G R H R C I G E N F
S.C.	CYP51	F **G** G G R H R C I G E H F
S.B.	CYP51	F **G** G G R H G C L G E P F
M.T.	CYP51	F **G** A G R H R C V G A A F
C.A.	CYP51	F **G** G G R H R C I G E Q F
C.A.	CYP51(G464S)	F **S** G G K H R C I G E Q F
Rat	CYP2B1	F **S** T G K R I C L G E G I
Human	CYP2C9	F **S** A G K R I C V G E A L
Human	CYP2E1	F **S** T G K R V C A G E G L
Rabbit	CYP2B4	F **S** L G K R I C L G E G I

FIG. 5. Amino acid sequences of P450s in the heme binding region. Alignment of CYP51 amino acid sequences from human, rat, *Saccharomyces cerevisiae* (S.C.), *Sorghum bicolor* (S.B.), *Mycobacterium tuberculosis* (M.T.) and *Candida albicans* (C.A.) and a comparison to the same region in CYP51(G464S) and various mammalian CYP isoforms. The conserved glycine and serine residues are depicted in bold.

protein of the azole compounds in resistant strains that reduced affinity was one mechanism that might be occurring. The substitution G464S occurs in a highly conserved region of CYP51 (Fig. 5), which encodes an ancestral function in the cytochrome P450 superfamily found in animals, plants fungi and recently *Mycobacteria* [20]. The heme thiolate ligand of the protein involves C470 and in some ways the finding of altered fluconazole affinity in the mutant G464S is surprising as molecular modeling based on the known structure of cytochrome $P450_{CAM}$ indicated the direct molecular interactions of the drug occur above the heme. The N-1 substituent of the drug was predicted to interact with the protein via aromatic interactions with F233 or F235 while the triazole N4 anchored the drug to the heme as a sixth ligand [21].

The data described here show the G464S substitution exerts an effect on azole sensitivity of cells containing this protein by a subtle modification. The heme environment of the protein was perturbed as shown by the altered Soret peak of the reduced carbon monoxide difference spectrum where a shift from 447 to 450 nm occurred. Although the azole binding Type II spectra did not show differences in terms of maxima/minima, a substantial difference was observed in affinity when the amounts required to reach saturation of the spectral change were investigated using equal amounts of wild-type and mutant protein. Fifteen times more fluconazole than CYP51(G464S) was required for saturation to be achieved unlike the wild-type protein where stoichiometric binding was observed reflecting very high affinity. Studies on enzyme activity revealed the mutant protein retained catalytic activity, but the IC_{50} confirmed that reduced affinity for fluconazole occurred due to the G464S substitution in CYP51.

No alteration in affinity of the substrate was observed between the mutant and wild-type proteins, but reduced activity was observed. The molecular interaction of substrate with CYP51 has been proposed to involve hydrogen bonding between T315 and sterol 3-OH [22]. It is possible this interaction still occurs, but that alterations in the active site involving the heme position results in different positioning of the 14α-methyl group relative to the oxygen bound above the heme. This would result in reduced efficiency during the monooxygenation reactions that result in demethylation as observed in the reduced maximal enzymatic rate of the mutant protein. The retention of catalytic activity reflects a resistance mechanism allowing continued production of ergosterol as we detected in strains containing the substitution G464S (unpublished observations).

The changes resulting in an alteration of the heme environment and fluconazole affinity cannot be fully explained until structures of wild-type and mutant CYP51 can be produced. A suitable hypothesis to explain the effects observed is that the heme is moved in some way in terms of location (up, down, or angle). The heme-binding domain of other cytochromes P450 can have either residue at this position so this change may also contribute to the differing regio- and stereospecificities of other enzymes besides CYP51. The recent production of a soluble and catalytically active *C. albicans* CYP51 [23] may assist the production of structural information as might experiments on the naturally occurring, soluble sterol 14α-demethylase of *Mycobacterium tuberculosis* [24].

The appreciation that fluconazole resistance can arise through altered affinity for *C. albicans* CYP51 means that a rational approach can be adopted to seek resistance-breaking therapeutic regimes which may differ for different mechanisms. Screens can now be made of compounds which may continue to inhibit mutant enzyme or which may have increased activity on mutant enzyme vis-à-vis wild type. In comparison with successful strategies used in antiviral therapy it may also be possible to utilize combination drugs to overcome resistance.

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