Sterol 14α-demethylase (P45051) is the target for azole antifungal compounds, and resistance to these drugs and antifungals is of significant practical importance. We undertook site-directed mutagenesis of the Candida albicans P45051 heterologously expressed in Saccharomyces cerevisiae to probe a model structure for the enzyme. The change T315A reduced enzyme activity 2-fold as predicted for the removal of the residue that formed a hydrogen bond with the 3-OH of the sterol substrate and helped to locate it in the active site. This alteration perturbed the heme environment, causing an altered reduced carbon monoxide difference spectrum with a maximum at 445 nm. The changes also reduced the affinity of the enzyme for theazole antifungals ketoconazole and fluconazole and after expression induced by galactose caused 4–5-fold azole resistance in transformants of S. cerevisiae. This is the first example of a single base change in the target enzyme conferring resistance to azoles through reduced azole affinity.

Sterol 14α-demethylase (P45051) participates in sterol biosynthesis, and is an essential requirement for yeast viability (1), and has been identified as the only cytochrome P450 found in animals, plants, and fungi, suggesting that it represents an ancient metabolic activity within the superfamily of P450 enzymes. Despite the presence of a human equivalent of the (2), selective inhibitors of the fungal enzyme have been developed as a central part of antifungal therapy. Such drugs are of increasing importance, since the incidence of fungal infection has risen dramatically due to immunocompromised conditions after organ transplantation, cancer chemotherapy, or for patients suffering from AIDS (3). Selective inhibitors have also been developed that act as agrochemical fungicides for phytopathogenic fungi but are tolerated by plants.

Most inhibitors of the enzyme contain an azole moiety, often a triazole. The N-4 (or N-3 of imidazole) can bind to the heme of the enzyme as a sixth ligand with the N-1 substituent group binding to the apoprotein (4). The development of resistance to fluconazole in >10% of late stage AIDS patients suffering candidiasis has stimulated interest on the mechanisms of resistance and how to diagnose and overcome this problem. It is known for a few isolates of Candida albicans that resistance can occur through phenomena related to decreased accumulation of drug in the cell (5, 6), although for other fungi suppressor mechanisms operate that change the 14α-methylated sterols accumulating under treatment (7, 8), and recently leaky P45051 mutants of Ustilagomaydis were identified that exhibited resistance, demonstrating altered target site as a mechanism of resistance in a congenic background (9). Previously, a P45051-defective strain, SG1, was observed to be azole-resistant, but such resistance can be attributed to the second defect it has in sterol Δ5,6-desaturation, which suppresses the effect of a chemical or genetic block at P45051 (10).

Information on the structure of eukaryotic P450s is lacking due to difficulties associated with crystallizing these micromolar proteins, but based on the known structure of soluble bacterial P450s, molecular modeling can be attempted. One such attempt was for C. albicans P45051 (11) and suggested interactions by the substrate with the apoprotein. The positioning of the substrate in the active site was modeled to consider the removal of the sterol 14α-methyl group, and a hydrogen bond between sterol C3-OH and Thr315 was predicted.

This paper presents results of experiments designed to examine the effect of mutation of this residue on activity and azole antifungal binding. Alteration to Ala by a single base substitution produced an altered protein exhibiting reduced activity. Furthermore, this altered protein had reduced affinity for fluconazole and ketoconazole and produced relative resistance in transformants of Saccharomyces cerevisiae.

EXPERIMENTAL PROCEDURES

Recombinant DNA Manipulations—Our previous studies have employed a yeast expression system to express the C. albicans P45051 using the S. cerevisiae GAL10 promoter in the vector YEp51 (12). Recombinant PCR‡ was employed to replace the codon 315 (ACT) with one encoding alanine (GCT). The following oligonucleotides were used as outside primers: 1) 5′-AAATTTGCTAAAGCTGCTTTGACTACT-3′, annealing to positions 429–456 of the C. albicans P45051 and preceding the endogenous BglII site present in the P45051 gene, and 2) 5′-TGGGATCATGTCGCTCTGTGAGTTTTT-3′, annealing to the 3′-end at positions 1098–1125 of the P45051 and containing the endogenous NsiI site present in the gene. Inside primers used in the PCR mutagen-

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thesis were 1) 5‘-ACTTCTGCTTCTGCTTCTGCTTGGTTC-3’, and 2) 5‘-GAACCAAGCAGAAGCAGAAGCAGAAGT-3’. In a first step, two separate PCR reactions 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 following PCR step using outside primer 1/outside primer 2. PCR reactions were performed on a Perkin-Elmer DNA thermal cycler with conditions consisting of an initial 5 cycles of 1 min denaturation at 94°C, an annealing step for 4 min at 48°C, and an extension step for 3 min at 70°C, followed by 25 cycles of a denaturation step for 1 min at 94°C, an annealing step for 2 min at 55°C, and an extension step for 3 min at 72°C. PCR was undertaken using Pfu polymerase (Stratagene). The resulting DNA fragment containing the T315A mutation was ligated into the YEp51: P45051 expression vector YEp51, allowing expression from the GAL10 promoter. bps, base pairs.

Strains and Culture Conditions for Heterologous Expression—The plasmid YEp51:P45051(T315A) was transformed into S. cerevisiae strain GRF18 (MATa leu2-3,2-112 his3-11,15 can1). Yeast transformants were grown at 28°C, 250 rpm with a 250-ml culture in 500-ml flasks. The media used consisted of Difco yeast nitrogen base with conditions consisting of an initial 5 cycles of 1 min denaturation at 94°C, an annealing step for 4 min at 48°C, and an extension step for 3 min at 70°C, followed by 25 cycles of a denaturation step for 1 min at 94°C, an annealing step for 2 min at 55°C, and an extension step for 3 min at 72°C. PCR was undertaken using Pfu polymerase (Stratagene). The resulting DNA fragment containing the T315A mutation was ligated into the YEp51: P45051 expression plasmid according to Fig. 1. Introduction of the mutation and maintenance of the authentic sequence was corroborated by DNA sequencing using Sequenase 2 (Amersham Corp.). All restriction enzymes and T4DNA ligase were obtained from Promega, and the recommended conditions for use were applied.

Strains and Culture Conditions for Heterologous Expression—The plasmid YEp51:P45051(T315A) was transformed into S. cerevisiae strain GRF18 (MATa leu2-3,2-112 his3-11,15 can1). Yeast transformants were grown at 28°C, 250 rpm with a 250-ml culture in 500-ml flasks. The media used consisted of Difco yeast nitrogen base without amino acids (1.54% w/v) supplemented with 100 mg/l histidine and 2% (w/v) glucose as an initial carbon source. Heterologous expression was induced when the glucose was exhausted at a cell density of approximately 10⁸ cells/ml. The culture was left a further 4 h before galactose was added to a concentration of 3% (w/v). After a 20-h 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 x g for 10 min using a bench centrifuge and enzyme preparation was at 4°C. The resulting supernatant was centrifuged twice at 10,000 x g and diluted with a 20% (v/v) glycerol solution to 25 mM potassium phosphate buffer. Protein concentrations were measured as indicated previously (14). Purification of C. albicans Sterol 14a-Demethylase—200 nmol of heterologously expressed C. albicans wild type and mutant P45051 were solubilized in 100 mM potassium phosphate buffer, pH 7.4, containing 2% (w/v) sodium cholate for 1 h at 4°C, respectively. Solubilized P450 was recovered after a 1-h centrifugation step at 100,000 x g and diluted with a 20% (v/v) glycerol solution to 25 mM potassium phosphate, 0.8% (w/v) sodium cholate. The supernatant was applied to an amino-octyl-Sepharose column equilibrated with a 10 mM potassium phosphate buffer, pH 7.4, and 0.8% (w/v) sodium cholate. The protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. The enzyme was purified by affinity chromatography on an amino-octyl-Sepharose column.
phosphate buffer, pH 7.4, containing 0.8% (w/v) sodium cholate. The column was washed with 100 ml of 10 mM potassium phosphate buffer, pH 7.4, containing 0.8% (w/v) sodium cholate, by a second wash containing a similar buffer containing 1.2% (w/v) sodium cholate, and a third wash with 100 ml of 100 mM potassium phosphate buffer containing 0.5% (w/v) sodium cholate. P45051 was eluted from the column with 100 mM potassium phosphate buffer, pH 7.4, containing 0.5% (w/v) sodium cholate and 0.3% (v/v) Tween 20. P45051-containing fractions were pooled and dialyzed overnight against 2 liters of 100 mM potassium phosphate buffer, pH 6.8, containing 0.3% (w/v) sodium cholate. The sample was then loaded onto a hydroxylapatite column equilibrated with 10 mM potassium phosphate buffer, pH 6.8. The column was washed with 10 mM potassium phosphate buffer, pH 6.8, and eluted using a 10–150 mM potassium phosphate gradient. P45051-containing fractions (assessed by reduced carbon monoxide difference spectroscopy) were pooled and concentrated using an Amicon Centricon 10 microconcentrator. Enzyme purity was assessed by SDS-polyacrylamide gel electrophoresis and specific content. Purified enzymes were stored at ~80 °C until use.

Determination of Sterol 14α-Demethylase Activity of the Microsomes—A solution of NADP⁺ (2 mg), glucose-6-phosphate (5 mg) and glucose-6-phosphate dehydrogenase (3 units) in 100 mM potassium phosphate buffer containing 0.1 mM EDTA, 1 mM glutathione, and 20% (v/v) glycerol (0.5 ml, pH 7.4) was incubated at 30 °C for 20 min. To this was added microsomal protein (approximately 10 mg), and the volume was made up to 1 ml with the above buffer. Following the addition of the 32-tritiated substrate (52 μg, 1.62 μCi in 10 μl of dimethylformamide; see for 3) 0.1-nl aliquots were removed (at intervals of 0, 5, 10, 30 and 60 min) and added to a mixture of dichloromethane (0.5 ml) and water (0.5 ml). The mixtures were immediately shaken and then centrifuged. The organic layer was discarded, further dichloromethane (2 × 0.5 ml) was added, and the above procedure was repeated. To the resulting aqueous phase was added charcoal, and the suspension was 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. These reactions were compared with turnover of purified reconstituted enzyme using equivalent substrate and P450 concentrations (method described below).

Determination of Sterol 14α-Demethylase Activity of the Purified Proteins—Each reaction mixture contained purified P45051 and P45051(T315A), respectively (0.2 nmol), and 1 unit of rabbit NADPH-cytochrome P450 reductase in a total volume of 50 μl. To this, 50 μg of dilauroylphosphatidylcholine was added, and the reaction volume was adjusted to 950 μl with 100 mM potassium phosphate buffer, pH 7.4. 32-Tritiated substrate was added, and the mixture was sonicated until a white suspension formed. NADPH was added at a concentration of 23 mM to the mixture to start the reaction. For determination of enzyme kinetics, varying concentrations of substrate were used. Extraction and assessment of radioactivity was carried out as described above.

Inhibition of Sterol 14α-Demethylase Activity—Sterol 14α-demethylase activity of the purified preparations of P45051 and P45051(T315A) was assayed as described above. Azole antifungals, ketoconazole, and fluconazole (Fig. 2), were added to the reaction mixtures, respectively, from 1000-fold stock solutions.

Inhibition of Growth—Minimum inhibitory concentrations were estimated following the inoculation of 5 × 10⁵ cells/ml in minimal medium containing 100 μg/ml histidine and varying doses of ketoconazole and fluconazole, respectively. Incubation was at 30 °C, 150 rpm in 60-ml sterile containers (Sterilin) using 2 ml of culture. Growth was determined by cell counts and measuring unit absorbance.

Difference Spectroscopy—Microsomal samples (4 × 10⁻³ ml) of P45051 and P45051(T315A), respectively, were placed in both sample and reference cuvettes (1-cm path length) of a Philips PU8800 spectrophotometer. Azole antifungals, ketoconazole, and fluconazole, dissolved in Me₂SO were added directly to the sample cuvette; the contents were mixed, and after 1 min the spectrum between 500 and 350 nm was recorded. By adding successive concentrations of test substance, the change in absorbance between the type II peak (420–427 nm) and the corresponding trough (390–410 nm) was related to the concentration of added azole antifungal. The maximum concentration of Me₂SO used (1% (v/v)) caused no change in the spectrum over the region scanned.

EPR Spectroscopy—EPR spectra were recorded with an updated Bruker ER 200D-SRC spectrometer equipped with an Oxford Instruments ESR900 liquid helium cooling system, an NMR gaussmeter, and a microwave frequency counter. Spectra were recorded at 5 K with a microwave power of 0.5 milliwatts at 9.6 GHz using 1.0 millitesla field modulation at 100 kHz.

RESULTS

Heterologous Expression of P45051 and P45051(T315A)—Previously (12), we have reported high level expression of C. albicans sterol 14α-demethylase in S. cerevisiae, with P45051 levels exceeding 1.5 μmol/liter of culture, which is higher than any other expression of heterologous P450 in the literature. The expression system 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 Thr²¹⁵ to Ala, involving a single base substitution of ACT → GCT, which is outlined in Fig. 1. S. cerevisiae strain GRF18 transformed with P45051(T315A) expressed levels of P450 comparable with wild type enzyme with up to 2.5 nmol of P450/mg of 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 P45051 was located at 448 nm. However, the CO-bound form of P45051(T315A) had the Soret absorption maximum at 445 nm (Fig. 3). It was noted that no peaks at 420 nm were obtained for either P45051 or P45051(T315A) (P420 is an inactive derivative of P450), indicating stable production of both proteins.

Purification of P45051 and P45051(T315A)—Table I summarizes the results of each stage of the purification procedure starting from the microsomal fractions of P45051. The methods employed permitted the purification of both P45051 and P45051(T315A) with a yield of about 50% and to a specific content of approximately 17 nmol/mg microsomal protein, consistent with a purified hemoprotein of the predicted M₉. Upon SDS-polyacrylamide gel electrophoresis, pure preparations gave single protein bands confirming homogeneity with an apparent monomeric M₉ = 55,000 (Fig. 4).

EPR Spectra of P45051(T315A)—Fig. 5 shows the EPR spectrum of microsomal P45051(T315A) together with the spectrum of a control sample not expressing C. albicans P45051. The clearest features are at g values 2.43, 2.27, and 1.92 in the spectrum from P45051(T315A); these are very similar to those reported for low spin ferric iron in various cytochrome P450s and in particular are essentially identical to the g values reported for low spin ferric iron in various cytochrome P450s.
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FIG. 3. Reduced carbon monoxide difference spectrum of microsomal cytochrome P45051(T315A). Shown are the reduced carbon monoxide difference spectra of microsomal fractions, prepared as described under “Experimental Procedures,” after the induction of heterologous expression in yeast transformants containing YEp51 alone (---) and YEp51:P45051(T315A) (——).

TABLE I
Purification of C. albicans P45051 heterologously expressed in yeast strain GRF18

A typical experiment for the purification of P45051 after its expression in S. cerevisiae is described. The protein was expressed to a very high level in the microsomal fraction and was purified in essentially one step after washing the AO-Sepharose column with 1.2% (w/v) sodium cholate as described under “Experimental Procedures.” The Tween 20 used to elute P450 from the AO-Sepharose column was removed during subsequent chromatography prior to reconstitution and spectral studies. Essentially the same results were obtained for purification of P45051 (T315A) as outlined in parentheses.

| Step          | Protein | Total content | Specific content | Yield |
|---------------|---------|---------------|-----------------|-------|
| Microsomes    | 80.0    | 200 (200)     | 2.5 (2.2)       | 100   |
| AO-Sepharose  | 11.03   | 160 (145)     | 14.5 (13.9)     | 80    |
| Bio-Gel HT eluate | 1.45 (1.87) | 25* (32*) | 17.30 (17.1) | 50 (64) |

* P450 content recovered following the application of 50 nmol to the HT column.

FIG. 4. Purification of C. albicans P45051 following heterologous expression in S. cerevisiae. SDS-polyacrylamide gel electrophoresis analysis of the various stages of P450 purification were undertaken on a 10% polyacrylamide-SDS gel and visualized with Coomassie Blue. Lane 1, molecular weight standards (92,500, 68,000, 45,000, and 29,000); lane 2, microsomal protein (15 μg); lane 3, amino-acyl-Sepharose eluate (0.8 μg of protein); lane 4, hydroxylapatite eluate (0.8 μg of protein). Essentially the same purification results were observed for P45051(T315A).

FIG. 5. EPR spectrum of ferric P45051(T315A) at liquid nitrogen temperature. EPR spectra were recorded for control microsomal extracts not expressing C. albicans P45051 (a) and for microsomal samples following heterologous expression of P45051(T315A) (b). P45051(T315A) concentration was 50 μM in buffer containing 0.4 M sorbitol, 50 mM Tris·HCl, pH 7.4. The microwave frequency of the spectrometer was 9.6 GHz using 1.0-mT millitesla field modulation at 100 kHz.

nmol of P450/min for a reconstituted assay containing purified C. albicans P45051 and rabbit liver NADPH cytochrome P450 reductase. The difference in activity between microsomal and pure enzyme may reflect a limitation in available reductase in the microsomal fractions. As expected, the demethylation activity was below the base line of detectability in microsomes from the host strain harboring the parent vector YEp51 (data not shown).

Growth Inhibition—S. cerevisiae transformants containing P45051 and P45051(T315A) showed no difference in their minimum inhibitory concentrations to ketoconazole and fluconazole following growth on yeast minimal medium containing 2% (w/v) glucose (Table III). Minimum inhibitory concentration values of 5 and 20 μM for ketoconazole and fluconazole, respectively, were obtained for transformants containing both P45051 and P45051(T315A) as well as for the host S. cerevisiae strain containing the empty expression cassette. However, minimum inhibitory concentration values determined following growth on galactose, resulting in induction of P450, revealed an in-
crease in resistance of the yeast to theazole antifungals in both yeast strains expressing P45051 and P45051(T315A) when compared with the control host (Table III). A respective 4- and 5-fold increase in resistance to ketoconazole and fluconazole was observed for the strain expressing wild type P45051 in contrast to a 20-fold increase in resistance to both azole antifungals that was observed following expression of P45051(T315A). No difference was observed in the P450 content following the expression of wild type P45051 and P45051(T315A) during growth on galactose, with specific contents of approximately 1.2 nmol of P450/mg of microsomal protein being observed following expression of both proteins. No detectable P450 was observed following growth of the host strain on glucose.

**TABLE II**

Enzymatic kinetic properties of purified P45051 and P45051(T315A)

| P450 preparation | $K_m$ ($\mu$M) | $V_{max}^a$ (pmol/min/nmol P450) |
|------------------|---------------|----------------------------------|
| P45051           | 29.4          | 147.0                            |
| P45051(T315A)    | 62.5          | 83.3                             |

$^a V_{max}$ values are expressed as pmol of substrate converted per min per nmol of P450.

**TABLE III**

Resistance studies in yeast transformants

| P450                  | Minimum inhibitory concentration without galactose | Minimum inhibitory concentration with galactose |
|-----------------------|-----------------------------------------------------|-----------------------------------------------|
|                       | Ketoconazole ($\mu$M) | Fluconazole ($\mu$M) | Ketoconazole ($\mu$M) | Fluconazole ($\mu$M) |
| P45051                | 5.0                   | 20.0                 | 20.0                   | 100.0                 |
| P45051(T315A)         | 5.0                   | 20.0                 | 100.0                  | 400.0                 |

The added azole antifungal and resulting in a spectral peak (420–427 nm) and a corresponding trough (390–410 nm) (17). The intensity of the resulting difference spectrum has been characterized previously for wild type P45051 and found to be proportional to the amount of the azole-bound form of the cytochrome (18). Both ketoconazole and fluconazole caused the type II spectral change in both P45051 and P45051(T315A), but the positions of the peaks were altered in the mutant compared with the wild type. The spectral peak was located at 425 nm for P45051(T315A) compared with 424 nm for wild type P45051; the trough was located at 409 nm for P45051(T315A) compared with 412 nm for wild type P45051. Alteration in the positions of the peaks and troughs following azole antifungal binding in P45051(T315A) indicates an alteration in the conformational environment around the P450 heme. The apparent affinities of both ketoconazole and fluconazole for P45051(T315A) were different when compared with the wild type P45051. Ketoconazole and fluconazole were found to bind stoichiometrically with wild type P45051, whereas both compounds only formed one-to-one complexes with P45051(T315A) at an approximately 3-fold excess of azole antifungal over P450 (Fig. 7, a and b). The results of the spectrophotometric titration were consistent with those of the inhibition experiments (Table IV), confirming that the difference in the inhibitory effect resulted from the difference in the affinities of both ketoconazole and fluconazole for P45051(T315A) compared with P45051.
TABLE IV
Summary of comparative inhibition by azole antifungals

| P450      | 14a-demethylase activity inhibition | Ketoconazole | Fluconazole |
|-----------|-----------------------------------|--------------|-------------|
| P45051    | 0.02 nmol                         | 0.03 nmol    |             |
| P45051(T315A) | 0.12 nmol             | 0.16 nmol    |             |

The catalytic activity of purified P45051 and P45051(T315A), respectively, was assayed by measuring the release of [3H]formic acid plus [3H]water during the conversion of [32,3H]3β-hydroxylanost-7-en-3-ol to its C-14 demethylated product, 4,4-dimethyl-5α-cholesta-7,14-dien-3β-ol. Fig. 8a shows the results of an experiment in which 0.2 nmol of purified P45051 and P45051(T315A), respectively, were inhibited with increasing amounts of ketoconazole. For P45051, the inhibition of enzymatic activity was dependent on azole concentration, and total inhibition of enzyme activity occurred at a concentration equimolar to that of P45051. However, for purified P45051(T315A) a 4-fold excess concentration of ketoconazole was observed to inhibit sterol 14a-demethylase activity. Essentially the same results were obtained with fluconazole (Fig. 8b); total inhibition of wild type P45051 activity occurred when equimolar amounts of fluconazole were added, whereas for P45051(T315A) 4-fold excess amounts ofazole were required for total inhibition. The results are summarized in Table IV, where similar differences in $K_i$ were observed between P45051 and P45051(T315A), the latter showing reduced affinity for the azole antifungals.

DISCUSSION

The investigation of the three-dimensional structure of P450s remains of importance due to their roles in the metabolism of foreign compounds (carcinogens, drugs, and pollutants) as well as housekeeping roles in endogenous biosynthesis. Of the latter, considerable focus is now placed on the CYP51 family, which was cloned and sequenced first in S. cerevisiae (1) and later in other fungi (e.g. C. albicans; Ref. 19), rats (20), humans (2), and, from unpublished evidence, in plants also.2 The ancestor of these enzymes may have represented one of the earliest functions of P450.

In the sequences of known sterol 14a-demethylase genes, residues corresponding to Thr315 are conserved. Table V shows the alignment of various sequences. This residue is contained in the I-helix of P450 when modeling from the P450 structures solved for soluble bacterial enzymes (for review, see Ref. 21). Molecular modeling of P45051 of C. albicans predicted that this residue could form a hydrogen bond to the 3-OH of sterol, helping to locate the sterol in the active site (11). Our mutagenesis and activity experiments support the validity of this model, since reduced activity and affinity were observed for the mutant enzyme. Gross conformational change would likely give rise to P420, the inactive form of the enzyme, and a drastically altered active site would be unlikely to retain the ability to undertake the highly specific stereo- and regiospecific monooxygenase activity toward the sterol 14a-methyl group. The absence of any perturbation of the EPR spectra of mutant compared to the wild type protein also supports this conclusion.

Leaky P45051 mutants have been identified as being azole-resistant in other laboratory studies on U. maydis (9) and prompted further study of the mutant enzyme. Alteration of the Thr315 residue to alanine also subtly disturbed the hemoprotein as indicated by the change in the Soret maximum to 445 nm from 448 nm. This change in conformation is presumably responsible for the change in azole affinity observed in biochemical and microbiological assays of inhibition. In other studies, the stoichiometric binding of ketoconazole and fluconazole to purified P45051 of C. albicans has been established (18), but the T315A protein exhibited reduced affinity. This resistant mutation should be anticipated to occur in natural populations exposed to azole antifungals, since it results from a single base change. For AIDS patients, a limited number of fluconazole-resistant Candida species have been evaluated so far. Two resistant isolates were shown to have reduced accu-

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2 F. Durst, personal communication.
mulation of drug within cells (5), which explained their phenotype, rather than changes in the target site or of other sterol biosynthesis suppressor mutants as characterized for *S. cerevisiae* (7). Examination of resistant strains has also indicated increased efflux of drug as a cause of resistance in a limited number of further clinical strains, although comprehensive examination of other possible mechanisms of resistance was not included (6). An observation that has been made in a clinical setting is the raising of minimum inhibitory concentrations in increments during the development of resistance (22), and it seems likely that in some situations alterations in the active site will play a role. For other fungi, alteration in P45051 sensitivity has been correlated with resistance in isolates from AIDS patients of *Cryptococcus neoformans* (23) and of *Aspergillus fumigatus* rather than the other possible causes of resistance. This is the first paper to demonstrate a molecular mechanism altering theazole affinity of the P45051 enzyme to make it more resistant.

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