Characterization of Saccharomyces cerevisiae CYP61, Sterol \( \Delta^{22} \)-Desaturase, and Inhibition by Azole Antifungal Agents*

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Cytochrome P-45061 (CYP61) was a cytochrome P-450 revealed during the yeast genome project when chromosome XIII was sequenced. Here we report on the properties of this second microsomal P-450 of vegetatively growing yeast. The enzyme kinetics associated with its endogenous role in sterol \( \Delta^{22} \)-desaturation revealed a \( K_m \) of 20.4 \( \mu \)M and a \( V_{\text{max}} \) of 2.9 nmol/min/nmol CYP61. The affinity of the enzyme for antifungal drugs was characterized to investigate its potential role in determining tolerance to these sterol 14α-demethylase (CYP51) inhibitors. Drug binding induced a type II spectral change, which became saturated at equimolar concentrations of azole drug and P-450. Fluconazole exhibited slightly reduced affinity in comparison to ketoconazole as indicated by carbon monoxide displacement. These and \( K_m \) determination for fluconazole (0.14 nm) revealed CYP61 to have a similar affinity to azole drugs when compared with data available for CYP51, and the implications for antifungal treatment were considered.

The cytochrome P-450 (CYP) superfamily are involved in a variety of monoxygenase reactions including xenobiotic and endogenous substrates. Genome projects are uncovering more genes encoding such proteins, and one such instance was cyp61 (ERG5) encoded on chromosome XIII of Saccharomyces cerevisiae (1). Previous studies on S. cerevisiae have identified a single form of P-450 during purification from microsomal fractions of vegetatively growing yeast. This P-450 performed a role in sterol 14α-demethylation (2), later associated with the gene, cyp51 (ERG11; Ref. 3). This is the only P-450 activity associated with a family found in plants, animals, and fungi (4). Other studies based on inhibition and co-factor characteristics indicated a further P-450-mediated activity was present in vegetative yeast (5). This was confirmed when such a protein was purified from microsomes obtained from a vegetatively growing strain with a cyp51 gene disruption (6). N-terminal amino acid sequence confirmed the protein to be CYP61 from chromosome XIII (1).

Inhibitors of CYP51 are of considerable commercial importance as antifungal compounds and selectively inhibit fungal CYP51 over the mammalian and plant counterparts (7). Because they may also bind to CYP61, a potential antifungal target, we characterized the affinity of this P-450 for two of the main drugs employed, ketoconazole and fluconazole, to consider the relative potency and potential contribution CYP61 may make to azole antifungal susceptibility.

EXPERIMENTAL PROCEDURES

Materials—Unless specified, all chemicals were obtained from Sigma (Poole, Dorset, UK). Ketoconazole was purchased from Janssen Pharmaceutica, and fluconazole was from Pfizer. Microsomes were prepared, and CYP61 was purified from semi-anaerobically grown cells of the S. cerevisiae strain DR2, which contains a gene disruption in cyp51 (\( \Delta^{14} \text{erg} \); Ref. 3). Rabbit NADPH-cytochrome P-450 reductase was a gift from Prof. M. Akhtar, University of Southampton. Ergosta-5,7-dienol was purified from a polyene-resistant erg5 mutant of S. cerevisiae (\( \Delta^{22} \)-desaturase defective) as described previously (6).

Reconstitution of Sterol \( \Delta^{22} \)-Desaturase Activity—The standard reaction mixture contained purified CYP61 (0.5 nmol), 1 unit of rabbit NADPH cytochrome P-450 reductase, and varying concentrations of ergosta-5,7-dienol dispersed in 80 nmol disphosphatidylcholine, and the reaction volume was adjusted to 950 \( \mu \)l with 100 mM potassium phosphate buffer, pH 7.2. NADPH was added at a concentration of 23 mM to the mixture to start the reaction. For experiments involving inhibition of activity, azole drug was added prior to NADPH. All reactions were incubated at 37 °C for 20 min in a shaking water bath. Reactions were stopped by the addition of 3 ml of methanol, and the sterols were extracted using 2 ml of pyrogallol in methanol and 2 ml of 80% (v/v) potassium hydroxide (in water), incubated at 90 °C for 2 h in a preheated water bath. After cooling the saponified mixture was extracted with 3 × 5 ml of hexane and dried under nitrogen. A Hewlett-Packard gas chromatograph/mass spectrometer was used to confirm sterol identities. An Ultra 1 capillary column was used (10 m × 0.2 i.d.) on a temperature program 50 °C (1 min) increased by 40 °C/min to 290 °C with a run time of 47 min. Injection port temperature was 280 °C (splitless), and the carrier gas was helium at 40 kilopascal. Trimethylsilylated derivatives of ergosta-5,7-dienol, and the \( \Delta^{22} \)-desaturated metabolite (ergosterol) were clearly separated as two distinct peaks (6). The conversion ratio was calculated from the areas of the two peaks, and the activity (nmol ergosterol formed/min) was obtained from the amount of ergosta-5,7-dienol added and the conversion ratio. Linear regression was used in double reciprocal plot analysis.

Difference Spectroscopy—Binding spectra were obtained for azole antifungals according to the method of Wiggins and Baldwin (8). Briefly, purified CYP61 (0.2 nmol/ml) was placed in both sample and reference cuvettes (1-cm path length) of a Philips PU8800/02 scanning spectrophotometer. Azole antifungals dissolved in Me\(_2\)So were added direct to the sample cuvette, the contents were mixed, and the spectrum was recorded between 350 and 500 nm. By adding azole antifungal, 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 azole antifungal added. The maximum concentration of Me\(_2\)So used (1% by volume) caused no change in the spectrum over the region scanned.

Carbon monoxide displacement studies of azole antifungal bound to CYP61 was monitored using equimolar concentrations of CYP61 (0.2 nmol/ml) and varying concentrations of azole antifungal. After 2 min the content of both cuvettes was reduced by the addition of sodium dithionite, and a base line was recorded between 400 and 500 nm. The contents of the sample cuvette were bubbled with carbon monoxide for 30 s. The cuvette was sealed, and the reduced difference spectra were recorded.

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RESULTS

Ergosta-5,7-dienol Metabolism by a CYP61 Reconstituted System—Ergosta-5,7-dienol was aerobically metabolized to ergosterol (Fig. 1A) by a reconstituted monooxygenase system containing CYP61 and NADPH cytochrome 450 reductase. The purified enzyme was shown to have a \(K_m\) of 20.4 \(\mu\)M for ergosta-5,7-dienol and a maximal enzymatic rate (\(V_{max}\)) of 2.9 nmol ergosterol formed/min/nmol CYP61. The identity of the sterol peaks in gas chromatography were confirmed by mass spectrometry and showed conversion of the sterol into ergosterol. Control experiments showed P-450 and NADPH dependence for the reaction.

Spectral Ligand Binding Studies on CYP61—The absolute absorption spectrum of CYP61 is shown in Fig. 2. The oxidized cytochrome absorbed at 553 and 532 nm with a Soret maximum at 417 nm, indicating that it was in the low spin state. The Soret peak of the reduced carbon monoxide (CO) complex was situated at 448 nm. Addition of theazole antifungals, ketoconazole and fluconazole (Fig. 1B), gave rise to typical type II difference spectra, on binding of N-3 of imidazole (ketoconazole) and N-4 of triazole (fluconazole) to the CYP61 heme, with absorption maximum and minimum at 428 and 410 nm, respectively. The increase in value of absorbance (peak maximum to peak minimum) measured after successive additions of ketoconazole and fluconazole showed that the spectral change was linearly dependent on the concentration of azole antifungal and was saturated when equimolar amounts of ketoconazole and fluconazole were added (Fig. 3). Fig. 4 shows the results of experiments to measure displacement of azole by CO. The concentration of ketoconazole required to inhibit CO binding to CYP61 by 50% (IC\(_{50}\)) was 0.2 nmol. The larger IC\(_{50}\) value of 0.5 nmol for fluconazole suggests that this compound was less able than ketoconazole to inhibit the formation of the CO-CYP61 complex.

Inhibition of Reconstituted CYP61 \(\Delta^{22}\)-Desaturation by Azole Antifungal—Fluconazole was compared for its inhibitory activity against reconstituted CYP61-mediated sterol \(\Delta^{22}\)-desaturation, and the results were compared with its inhibitory activity against the target enzyme, Candida albicans CYP51 (sterol 14a-demethylase). Fluconazole was shown to be a competitive inhibitor of CYP61 as shown in Fig. 5. The \(K_i\) for fluconazole inhibition of CYP61 was calculated to be 0.14 nm.
DISCUSSION

Previously we reported the purification of a second P-450 of vegetatively growing yeast, CYP61, from a strain containing a gene disruption in cyp51 (5). The enzymological properties of CYP61 in sterol \( \Delta^{22}\)-desaturation were characterized here for the first time with reference to CYP51 (sterol 14\( \alpha\)-demethylase) previously purified from vegetatively growing yeast (2). We also investigated the affinity of CYP61 for azole antifungals because this enzyme may contribute to overallazole tolerance or to resistance of fungi, if possessing a high affinity. Resistance is a major problem in agriculture and in the clinic where fluconazole resistant Candida sp. occur in >10% of late stage AIDS patients. It may represent a potential target for new antifungal development in the mounting crisis of increased fungal disease (7).

In undertaking sterol \( \Delta^{22}\)-desaturation the substrate affinity constant of 20.4 \( \mu\)M and maximum enzymatic rate of 2.9 nmol/min/nmol P-450 observed for CYP61 were typical of other P-450s mediating endogenous reactions including CYP51-mediated sterol 14\( \alpha\)-demethylation, where a \( V_{\text{max}} \) of 9 nmol/min/nmol P-450 was observed (9).

The absolute absorption spectrum of CYP61 was in general similar to CYP51 with the hemoprotein in a low spin state and the reduced carbon monoxide complex giving a maximum at 448 nm. An exception was in the visible region, where the peaks at 553 and 532 nm are in contrast with those reported for sterol 14\( \alpha\)-demethylase (CYP51), which gave peaks at 570 and 534 nm (2).

Azole binding and inhibition was examined with reference to published data. The ability of ketoconazole (10) and fluconazole\(^1\) to inhibit CO binding to C. albicans CYP51, was examined and gave IC\(_{50}\) values of 0.1 and 0.3 nmol, respectively. Using the same quantity of enzyme the corresponding IC\(_{50}\) values for CYP61 were only slightly larger at 0.2 and 0.5 nmol, respectively. These findings reveal only slightly reduced sensitivity of CYP61 compared with CYP51 forazole antifungal binding. Using this assay on a range of plant and mammalian enzymes, IC\(_{50}\) values several orders of magnitude higher were obtained (11). This relatively high affinity for azole antifungals was further supported by studies on the inhibition of activity. Competitive inhibition was observed for fluconazole and a \( K_{i} \) of 0.14 nm. This value compares with 0.10 nm for C. albicans CYP51.\(^1\) Thus inhibitors of CYP51 probably act on CYP61 at subminimum inhibitory concentrations for yeast, but with growth arrest 14\( \alpha\)-methylated sterols predominate in which many subsequent reactions, including \( \Delta^{22}\)-desaturation, of the ergosterol pathway are blocked. Other inhibitors of CYP51 have been observed to inhibit CYP61 (12), and in some treatments of the cereal pathogen Rhynchosporium secalis these effects seem to predominate and correlate with growth arrest.\(^2\)

Thus CYP61 may represent an antifungal target, although gene disruption has indicated that for growth of yeast on glucose under laboratory conditions it is not essential (13).

Sterol \( \Delta^{22}\)-desaturase is an ancient P-450 activity reflected in the presence of such desaturation in some plant sterols as well as in fungal sterols. The plant activity may well be mediated by a member of the same P-450 family, as for sterol 14\( \alpha\)-demethylase undertaken by members of the CYP51 family in animals, plants, and fungi. It might be anticipated that further studies on CYP61 will cast light on the evolution of the P-450 superfamily and that this enzyme may have arisen from CYP51 following a gene duplication event some time after the separation of plants and fungi from animals. Perhaps this was the first such development in plant and fungal P-450 evolution, and subsequent further divergence then occurred.

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