Crystal Structure of the Mycobacterium tuberculosis P450 CYP121-Fluconazole Complex Reveals New Azole Drug-P450 Binding Mode

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Azole and triazole drugs are cytochrome P450 inhibitors widely used as fungal antibiotics and possessing potent antymycobacterial activity. We present here the crystal structure of Mycobacterium tuberculosis cytochrome P450 CYP121 in complex with the triazole drug fluconazole, revealing a new azole heme ligation mode. In contrast to other structurally characterized cytochrome P450azole complexes, where the azole nitrogen directly coordinates the heme iron, in CYP121 fluconazole does not displace the aqua sixth heme ligand but occupies a position that allows formation of a direct hydrogen bond to the aqua sixth heme ligand. Direct ligation of fluconazole to the heme iron is observed in a minority of CYP121 molecules, albeit with severe deviations from ideal geometry due to close contacts with active site residues. Analysis of both ligand-on- and -off structures reveals the relative position of active site residues derived from the I-helix is a key determinant in the relative ratio of on and off states. Regardless, both ligand-bound states lead to P450 inactivation by active site occlusion. This previously unrecognized means of P450 inactivation is consistent with spectroscopic analyses in both solution and in the crystalline form and raises important questions relating to interaction of azoles with both pathogen and human P450s.

Fungal infections are successfully treated with azole and triazole drugs that inhibit fungal cytochromes P450 (P450s). A major target is the sterol demethylase (CYP51) and inhibition prevents synthesis of the membrane sterol ergosterol from lanosterol (1). This compromises membrane integrity and induces fungal cell lysis. Newer generation azoles and triazoles (e.g. fluconazole and voriconazole) have less severe interactions with human P450s and are used systemically as well as topically (2). Moreover, azoles are potent inhibitors of cell growth in mycobacteria and other actinobacteria (3). Furthermore, recent studies indicate that azole drugs have antitubercular activity in mice (4, 5). These genera have in common a high proportion of P450s, suggesting that azoles exert effects by inhibiting one or more P450 isoforms critical to viability or growth (6). In the pathogen Mycobacterium tuberculosis (Mtb), several azoles have been shown to bind the CYP51 and (more tightly) to the CYP121 P450 (7, 8). Currently, structures of three P450-antifungal azole complexes are available, showing direct coordination of heme iron by azole nitrogen in the case of Mtb CYP51 with fluconazole, Saccharopolyspora erythrae P450 eryF (CYP107A1) with ketoconazole, and rabbit microsomal CYP2B4 with bifonazole (9–11). Understanding molecular interactions of azoles in P450 active sites provides a route for designing more specificazole-based inhibitors and for rationalizing (and avoiding) development of drug resistance (12). However, if assuming a similar azole-nitrogen heme iron ligation occurs in Mtb CYP121, the tight binding of various azoles to Mtb CYP121 cannot easily be explained on the basis of the ligand-free CYP121 structure, given the constrained environment around the sixth heme coordination space (7). Thus, we considered it important to obtain an atomic structure of an azole complex of this P450 given our previous studies that indicated CYP121’s extremely tight binding of antifungal azoles (8) and since azole binding mode could not be readily rationalized on the basis of the ligand-free structure.

In work leading up to the crystallization of the fluconazole complex of CYP121, we established that the previously identified crystallization conditions are incompatible with co-crystallization or soaking experiments due to the low solubility of available tight-binding azole antifungals. Thus, to study binding mode of fluconazole to Mtb CYP121, we have defined new conditions compatible with the crystallization of CYP121 in both ligand-free and fluconazole-bound forms and have determined the Mtb CYP121 structure in presence/absence of fluconazole to 1.6 Å/1.9 Å, respectively. The data reveal an unprecedented mode of fluconazole coordination of the
CYP121—Fluconazole Structure

| TABLE 1 | Data collection and final refinement statistics |
|---------|-----------------------------------------------|
| Crystal | CYP121 | CYP121-Fluconazole |
| Space group | P2₁2₁2₁ | P2₁2₁2₁ |
| Diffraction limit (Å) | 15–1.60 (1.65–1.60) | 15–1.90 (1.95–1.90) |
| Reflections | 314231 | 166629 |
| I/σI (%) | 11.3 (1.8) | 9.4 (1.81) |
| Rmerge (%) | 5.5 (43.2) | 8.7 (40.5) |
| Completeness (%) | 98.5 (93.8) | 89.9 (90.8) |
| Rfactor | 16.4/21.0 (20.6/28.5) | 16.7/22.6 (21.1/28.9) |
| Root mean square deviation bond lengths (Å) | 0.015 | 0.017 |
| Root mean square deviation bond angles (°) | 1.534 | 1.601 |
| Average B-factor (Å²) | 23.5 | 26.8 |
| Protein Data Bank code | 2IJ5 | 2IJ7 |

CYP121 heme iron via a bridging water molecule and highlight the importance of the position of I helix residues in controlling the nature of heme iron coordination by fluconazole in the P450.

MATERIALS AND METHODS

**CYP121 Preparation and Spectroscopic Analysis**—CYP121 enzyme was expressed and purified as described previously (8). Fluconazole-bound CYP121 was prepared by dissolving solid fluconazole into the protein solution in 50 mM HEPES buffer, pH 7.0, to a saturating concentration of ∼5 mM. Binding was monitored by absorption spectroscopy in 0.1-cm path length quartz cells, following the type II spectral shift induced on interaction with the drugs. Protein concentrations were ∼250 μM for absorbance, EPR, magnetic circular dichroism (MCD), and 5 mg/ml for crystallography, unless otherwise stated. MCD samples were prepared in 50 mM HEPES/D₂O buffer, pH⁺ 7.0, containing deuterated glycerol (50% v/v). UV-visible absorption spectra were recorded on a Cary UV-50 Bio scanning spectrophotometer using 1- or 0.1-cm path length quartz cells.

X-Band EPR spectra were recorded on a Bruker ER-300D series electromagnet and microwave source interfaced to a Bruker EMX control unit and fitted with an ESR-9 liquid helium flow cryostat from Oxford Instruments and a dual mode electromagnet and microwave source interfaced to a Bruker EMX control unit from Bruker (ER-4116DM). Quantification of S = ½ species was carried out by comparison with the spin standard Cu²⁺ (EDTA) using the method of Aasa and Vanngard (13). Spectra of crystal slurries were taken using reduced volume EPR tubes.

Magnetic circular dichroism spectra were recorded using the Jasco J730 model in the near-infrared region. An Oxford Instruments split coil superconducting solenoid SM-4 was used to generate a field of 5 tesla and a temperature of 4.2 K. Room temperature measurements were carried out using an Oxford Instruments SM-1 superconducting magnet with an ambient bore. MCD intensities are plotted as Δε (m⁻¹ cm⁻¹) at low temperature and Δε/H (m⁻¹ cm⁻¹ T⁻¹) at room temperature. Room temperature MCD samples were prepared in 50 mM HEPES/D₂O buffer, pH⁺ 7.0. Deuterated glycerol (50% v/v) was added to the buffer for low temperature measurements.

Crystallographic Methods—X-ray diffraction data were collected from single crystals for the fluconazole complex and the fluconazole-free crystals at station ID 14.1, European Synchrotron Radiation Facility, Grenoble, France. Data were reduced and scaled using DENZO and SCALEPACK (14). The structure was solved using molecular replacement program AMoRe (15) with the wild type structure as search model (Protein Data Bank code 1N40). Atomic coordinates and B-factors were refined using the maximum likelihood-based Refmac5 (16). Data collection statistics and final refinement parameters can be found in Table 1. Structure factors and final coordinates are deposited in the Protein Data Bank with codes 2IJ5 (ligand-free form) and 2IJ7 (fluconazole-bound).

RESULTS AND DISCUSSION

Overall CYP121 Structure—Due to the extremely low solubility of the available azole antifungals in the previously identified CYP121 crystallization conditions, new crystallization conditions compatible withazole binding were determined. The new crystal form obtained contains six molecules in the asymmetric unit, and co-crystallization under saturating fluconazole concentrations reveals fluconazole bound in five out of the six molecules (molecules A–E). For all six monomers, there is no significant difference in overall conformation between the fluconazole-bound and fluconazole-free crystal structures. Furthermore, the conformation of the five fluconazole-binding monomers is highly similar to the conformation observed in the previously determined ligand-free CYP121 structure (Fig. 1A). In contrast, CYP121 molecule F has a conformation distinct from both the previously determined CYP121 structure and that of the remaining five molecules for residues 78–102 (the C-C’ helix region). In molecule F, Met-86 fills most of the active site cavity and thus obstructs binding of fluconazole or other compounds in the vicinity of the heme group (Fig. 1B). Given the lack of any clearly identifiable substrate-binding pocket near the heme group, it is unclear whether this “closed” conformation represents a catalytically relevant state. Crystal packing likely influences the preference for this closed conformation as...
CYP121-Fluconazole Structure

Active Site Structure of the CYP121-Fluconazole Complex—
Electron density for bound fluconazole could be clearly identified in each of the five monomers displaying the “open” conformation. Surprisingly, two distinct fluconazole conformations were observed, with varying occupancies for each conformation in the individual active sites. Conformation 1 involves heme ligation distinct from the direct ligation that previously has been observed in other P450 azole complexes (Fig. 2). In this case, one of the fluconazole triazole groups binds to the water sixth heme ligand (water W1) in addition to forming a hydrogen bond with a second water molecule (water W2) that is, in turn, hydrogen-bonded to a heme propionate (Fig. 2E). The remainder of the fluconazole molecule is bound predominantly via hydrophobic interactions with residues Met-52, Val-78, Val-82, Val-83, and Phe-168, in addition to interactions with the heme macrocycle. In addition, there is a water-bridged hydrogen bond (via W3) between the fluconazole hydroxyl group and the main chain oxygen of Thr-229 and a direct contact between the second triazole group and Gln-385 and Thr-77 (Fig. 2E). While fluconazole bound to molecules B and C solely adopts this conformation, a mixture of two conformations is observed in the other active sites (relative occupancies were determined by correlation of the average B-factors for each conformation with those of nearby residues in the active site; values can be found in Table 2). In these cases, a dual conformation is only observed for the ligand atoms, while protein-derived atoms in the active site occupy a single conformation. Despite the partial occupancy of conformation 2, omit electron density maps calculated with only conformation 1 present in the model allowed unambiguous identification of the conformation 2 binding mode (Fig. 2). The relative occupancy of the individual conformations was estimated and modeled so that the average B-factors of both conformations resembled the average active site B-factors. Unlike the first conformation, the second conformation involves direct ligation of heme iron via one of the triazole moieties, similar to that observed for the aforementioned CYP51, CYP107A1, and CYP2B4azole complexes (9–11). In addition to hydrophobic interactions made with the heme macrocycle, Thr-229, Phe-168, and Phe-280, there is a direct hydrogen bond between the non-ligating triazole and Arg-386, in addition to a water-mediated hydrogen bond between the fluconazole hydroxyl group and a heme propionate. This conformation is very similar to the geometry observed for the M. tuberculosis CYP51 fluconazole complex (9).

Conformation 2 Deviates from Ideality—Although direct coordination of CYP121 heme iron by fluconazole is observed in a proportion of the molecules, there are significant differences in the position and conformation of the fluconazole ligand by comparison with other P450-azole complexes. As for small molecule models of azole-ligated hemes or His-ligated hemoproteins (e.g. Ref. 17), the (tria)azole plane is expected to be perpendicular with the porphyrin macrocycle and the ligating nitrogen group linearly arranged with the iron and cysteinate sulfur atoms (Fig. 2). However, the observed direct fluconazole-CYP121 ligation deviates markedly from ideality. The triazole-ligating nitrogen atom is ~0.7 Å away from a position along the iron-sulfur axis, while the triazole cycle is at a 25° angle with that axis. Close contacts of the ligating triazole group with the side chains of both Ala-233 and Ser-237, both residues from the I-helix positioned immediately above the heme plane, clearly block the triazole moiety from occupying a near-ideal ligation pattern. There is a strong correlation between estimated occupancy for the direct-ligation conformation and the position of the I helix (as judged by the distance between residues Ala-233 and Ser-237 with the iron; see Table 2) with the most extreme cases being CYP121 monomers B and C, which have no detectable conformation 2. In the available P450 heme-(tria)azole complex structures (8–10), a similar relationship between ligand geometry and the relative position of I helix residues can be discerned (Table 3). The CYP121-fluconazole complex represents the most extreme deviation from ideal geometry, while the geometry observed for the CYP2B4 bifonazole complex (10) is near-ideal, due to the large distance between I helix residues and bound bifonazole molecule.
Spectroscopy of CYP121 Fluconazole Binding—Structural data are consistent with those from UV-visible spectroscopy. Typically, distal coordination of P450 ferric heme iron by an azole leads to shift of the major (Soret) absorbance band to ~424–425 nm. In CYP121, the Soret for the fluconazole complex is centered at ~422 nm, suggesting an altered ligation mode and the formation of a low spin heme iron complex that may not involve exclusively direct iron-nitrogen ligation (Fig. 3A). The fact that normal saturation behavior is observed for the binding of fluconazole to CYP121 (i.e. a hyperbolic dependence of observed spectral shift on ligand concentration, reaching a defined plateau), as well as the obvious lack of any significant amount of ligand-free species contributing to the final spectrum (see Fig. 3A), appears to rule out incomplete ligand binding as the origin of the observed 422 nm Soret shift.

EPR spectra of ligand-free CYP121 (Fig. 3B) have g-values at 2.48 (g_x), 2.25 (g_y), and 1.90 (g_z), as reported previously (6), and are comparable with those previously reported for other P450s, including the M. tuberculosis CYP51 (sterol demethylase) enzyme (2.44, 2.25, and 1.91). Thus, the spectrum of CYP121 in the absence of fluconazole has a typical rhombic triplet of signals arising from low spin cysteinate-coordinated ferric heme iron. The g_z feature, the best resolved of the three features, is broad and shows some structure. This suggests a degree of heterogeneity within the low spin heme. On binding of fluconazole to CYP121, a rhombic spectrum is obtained that is shifted and sharpened with respect to the ligand-free enzyme. The g-values for this complex are 2.45, 2.26, and 1.90 (Fig. 3B). This shift is unexpected, and typical g_z values for histidine- or imidazole-coordinated P450s lie in the range 2.65–2.50 (18). In addition, peak widths are narrower, and amplitudes are more intense than for the ligand-free CYP121. The EPR spectral features are thus consistent with a complex that has a substantial proportion of heme iron axially coordinated by cysteinate and an oxygen donor ligand. The g_z values for such complexes in other P450s are typically in the range from ~2.43 to 2.48 (e.g. 2.46 for the acetate complex of the Pseudomonas putida camphor hydroxylase P450 cam [CYP101A1]) (18).

MCD spectra were also recorded for the CYP121-fluconazole complex to provide further evidence supporting the novel coordination state observed in the crystals of the complex (Fig. 3C). MCD spectra of heme proteins are dominated by porphyrin π-π* transitions, which are perturbed by the heme iron and are therefore characteristic of the oxidation state (ferrous, ferric, ferryl) and spin state (low, high) of the heme iron. In the ferric state, ligand-to-metal charge transfer bands, which are weak and obscured in absorption spectra, are readily observed. Low spin ferric hemes give rise to a positive ligand-to-metal charge transfer feature (CT_{ls}) from the porphyrin-toiron d-orbitals in the near-IR region of the spectra. This band is sensitive
to the energies of the d-orbitals and shifts with changing heme ligation. The position of the band maximum can be used to assign heme ligands (20, 21). Both low temperature (4.2 K) and room temperature MCD spectra of the CYP121-fluconazole complex were recorded to obtain detailed data for the low spin complex and to enable ligand assignment. As a prelude to this experiment, it was established that the UV-visible spectrum of the CYP121-fluconazole did not change in the presence of the glassing agent (deuterated glycerol), required for work at the low temperature.

The room temperature MCD spectra (Fig. 3B) reveal a clear shift and decrease in intensity on fluconazole binding, where we assign the fluconazole bound form to be one in which fluconazole is mainly coordinated to heme iron via a water molecule. While we cannot rule out that a minor proportion of the sample is directly coordinated, the corresponding EPR spectrum (Fig. 3B) reveals a single species distinct from the ligand-free enzyme and proposed to be one in which fluconazole is coordinated via water. However, upon addition of 50% glycerol, two distinct low spin species are observed, the minor one of which (\(g_z = 2.5\)) we assign as containing a mixture of ligand-free and CYP121 with heme iron both directly and indirectly coordinated by fluconazole. The steric constraints imposed by the position of the I helix (e.g., Refs. 22 and 23) in CYP121, it becomes clear from our structural data that the positioning of the I helix is crucial with respect to controlling the mode of binding of bulky azole drugs. The steric constraints imposed by the position of the I helix reveal a new and unexpected mode of binding for fluconazole to heme iron in this system, which occurs via a bridging water molecule

### Table 2

| Molecule                  | B   | C   | D   | E   | A   |
|---------------------------|-----|-----|-----|-----|-----|
| Iron Ala-233 Cβ (Å)       | 5.44| 5.58| 5.78| 5.89| 5.49|
| Iron Ser-237 OH (Å)       | 4.40| 4.52| 4.63| 4.77| 4.71|
| Iron Ser-237 Ca (Å)       | 6.26| 6.39| 6.49| 6.56| 6.50|
| Iron Ala-233 Ca (Å)       | 6.14| 6.20| 6.38| 6.48| 6.49|
| Approximate occupancy ligated via water (%) | 100 | 100 | 70  | 50  | 50  |
| Approximate occupancy direct ligation (%) | 0   | 0   | 30  | 50  | 50  |

### Table 3

A comparison of active site stereochemistry for selected P450-azole complex structures

| Cytochrome P450 | CYP121 | CYP1 | CYP119 | CYP2B4 | CYP2B4 |
|-----------------|--------|------|--------|--------|--------|
| Resolution (Å)  | 1.9    | 2.2  | 1.9    | 1.9    | 2.3    |
| Ligand\(^a\)    | Fluc   | Fluc | 4-Phe  | 4-Cl   | BiFo   |
| Protein Data Bank code | 2IJ7 | 1EA1 | 1F4U   | 1SUO   | 2BDM   |
| Iron Ala-233 Cβ (Å) | 5.49 | 6.24 | 7.27   | 4.59   | 10.72  |
| Iron Ser/Thr-237 OH (Å) | 4.71 | 5.26 | 5.14   | 6.03   | 5.55   |
| Iron Ser/Thr-237 Ca (Å) | 6.50 | 7.30 | 7.38   | 7.40   | 7.66   |
| Iron Ala-233 Ca (Å) | 6.49 | 6.11 | 6.72   | 5.46   | 10.65  |
| Approximate offset\(^b\) (Å) | 0.7  | 0.3  | 0.2    | 0      | 0      |
| Angular deviation\(^c\) (°) | 25   | 15   | 20     | 15     | 0      |

\(^a\) Fluc = fluconazole, BiFo = bifonazole, 4-Phe = 4-phenylimidazole, 4-Cl = 4-(4-chlorophenyl)imidazole.

\(^b\) The approximate shortest distance between the ligation azole nitrogen and the line connecting the iron with the sulfur ligand.

\(^c\) Angular deviation: the smallest angle between the azole plane and the line connecting the iron with the sulfur ligand.

### Conclusion

The CYP121-Flu crystal structure contains a mixture of unliganded (monomer E, ~15%), indirectly liganded (approximately 60%) and a proportion of directly ligated CYP121 (~25%). An EPR spectrum from CYP121-Flu complex crystals indeed reveals multiple species (Fig. 3B) and can be interpreted as containing a mixture of ligand-free and CYP121 with heme iron both directly and indirectly coordinated by fluconazole.
FIGURE 3. Spectroscopic features of the CYP121-fluconazole complex. A, the main panel shows the UV-visible absorption spectrum of ligand-free CYP121 (black, Soret maximum at 416.5 nm, \(-23 \mu M\) P450) alongside that for the fluconazole complex (red line, Soret at 421 nm). B, in the top panel, the EPR spectrum obtained for solution state ligand-free CYP121 (black) is overlaid with those for fluconazole-bound forms (red without glycerol; blue line in presence of 50% v/v glycerol) of CYP121. Protein concentration was 255 \(\mu M\). EPR conditions were as follows: microwave frequency, 9.67 GHz; power, 2 milliwatts; temperature, 10.0 K; modulation amplitude, 10 G. g-values are detailed in the appropriate color code. In the lower panel, the corresponding EPR spectrum obtained for a microcrystalline suspension of CYP121-fluconazole complex is shown. C, near-infrared MCD spectra of CYP121 at 5 tesla, collected at room temperature (upper panel) and 4.2 K (lower panel). Samples were prepared as described under “Materials and Methods.” The room temperature spectra were collected for ligand-free enzyme (black line) as well as for fluconazole-bound enzyme (red line). The liquid nitrogen temperature spectrum of the corresponding fluconazole-bound sample reveals major features relating to the heme iron coordination at 1130 nm (assigned to azole coordination via water) and a shoulder at 1180 nm (assigned to direct coordination of the azole to the heme iron).
molecule. Spectroscopic studies both in solution and on the crystal complex are consistent with this finding and indicate that this indirect binding mode of fluconazole represents a high affinity state occupied in both crystal and solution states of the enzyme. These findings have major implications for understanding the interactions of this antifungal drug class with P450 heme and for the accurate modeling of protein-azole interactions that may be required for de novoazole drug design. Moreover, the novel binding mode also has clear relevance with respect to rationalizing azole drug resistance and its molecular determinants (e.g. Refs. 1 and 19). In conclusion, our data reveal an unexpected and novel binding mode for a clinically important drug class with its P450 protein target.

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