Domain-Swap Dimerization of *Acanthamoeba castellanii* CYP51 and a Unique Mechanism of Inactivation by Isavuconazole

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**ABSTRACT**

Cytochromes P450 (P450, CYP) metabolize a wide variety of endogenous and exogenous lipophilic molecules, including most drugs. Sterol 14α-demethylase (CYP51) is a target for antifungal drugs known as conazoles. Using X-ray crystallography, we have discovered a domain-swap homodimerization mode in CYP51 from a human pathogen, *Acanthamoeba castellanii* CYP51 (AcCYP51). Recombinant AcCYP51 with a truncated transmembrane helix was purified as a heterogeneous mixture corresponding to the dimer and monomer units. Spectral analyses of these two populations have shown that the CO-bound ferrous form of the dimeric protein absorbed at 448 nm (catalytically competent form), whereas the monomeric form absorbed at 420 nm (catalytically incompetent form). AcCYP51 dimerized head-to-head via N-termini swapping, resulting in formation of a nonplanar protein-protein interface exceeding 2000 Å² with a total solvation energy gain of −35.4 kcal/mol. In the dimer, the protomers faced each other through the F and G α-helices, thus blocking the substrate access channel. In the presence of the drugs clotrimazole and isavuconazole, the AcCYP51 drug complexes crystallized as monomers. Although clotrimazole-bound AcCYP51 adopted a typical CYP monomer structure, isavuconazole-bound AcCYP51 failed to refold 74 N-terminal residues. The failure of AcCYP51 to fully refold upon inhibitor binding in vivo would cause an irreversible loss of a structurally aberrant enzyme through proteolytic degradation. This assumption explains the superior potency of isavuconazole against *A. castellanii*. The dimerization mode observed in this work is compatible with membrane association and may be relevant to other members of the CYP family of biologic, medical, and pharmacological importance.

**SIGNIFICANCE STATEMENT**

We investigated the mechanism of action of antifungal drugs in the human pathogen *Acanthamoeba castellanii*. We discovered that the enzyme target (*Acanthamoeba castellanii* sterol 14α-demethylase (AcCYP51)) formed a dimer via an N-termini swap, whereas drug-bound AcCYP51 was monomeric. In the AcCYP51-isavuconazole complex, the protein target failed to refold 74 N-terminal residues, suggesting a fundamentally different mechanism of AcCYP51 inactivation than only blocking the active site. Proteolytic degradation of a structurally aberrant enzyme would explain the superior potency of isavuconazole against *A. castellanii*.

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**Introduction**

*Acanthamoeba* is a water- and soil-dwelling amoeba and an opportunistic pathogen of clinical interest. It is responsible for several diseases in humans, involving infections of the eye, brain, and skin (Marciano-Cabral and Cabral, 2003). *Acanthamoeba* has two distinct stages: trophozoite and cyst (Siddiqui and Khan, 2012). Sterol 14α-demethylase (CYP51) in *Acanthamoeba castellanii* is an essential enzyme in the biosynthesis of ergosterol, a functional analog of cholesterol in mammalian cells (Lamb et al., 2015; Thomson et al., 2017). CYP51 is a validated drug target in fungi and emerging drug target in the eukaryotic human pathogens (Choi et al., 2014b), including *Trypanosoma cruzi* (Choi et al., 2013, 2014a, b; Calvet et al., 2014; Vieira et al., 2014a, b), *Naegleria fowleri* (Debnath et al., 2017; Zhou et al., 2018), and *Acanthamoeba*. **CYP51**...
Despite these biophysical and biochemical observations, the luminescence resonance energy transfer (Davydov et al., 2015) was demonstrated for CYP3A4, CYP3A5, and CYP2E1 by homo- and hetero-oligomerization in microsomal membranes (Praporski et al., 2009). Homodimerization for the drug-metabolizing CYP2C8 was demonstrated by cysteine-scanning mutagenesis and 2D microbalance and atomic force microscopy (Praporski et al., 2005). Homodimerization in intact cellular membranes was suggested for the steroidogenic CYP17 and CYP19 by fluorescence resonance energy transfer and structures (Reed and Backes, 2017). As we discovered subsequently, the dimerization occurred via a domain-swap mechanism through the exchange of the N-terminal regions between two protomers. Domain swapping has not been reported in CYP protein family P450 previously. In this article, we use abbreviation CYP to denote a P450 enzyme of higher eukaryotes, when expressed heterologously, tend to form random aggregates in solution, and enzymes of lower eukaryotes, when expressed heterologously in cell-based assays (Lamb et al., 2015; Martin-Navarro et al., 2015; Thomson et al., 2017). However, the activity of isavuconazole against proliferating trophozoites is superior to both standard anti-Acanthamoeba therapy and other conazole drugs (Shing et al., 2020). Depending on the A. castellanii strain, isavuconazole potency varies in different strains from 26 nM (MEE1 0184) to 4.6 nM (Ma) to <1 nM (CDC:V240) (Shing et al., 2020). Against the A. castellanii Ma strain, isavuconazole potency (EC50 of 4.6 nM) was one order of magnitude higher than that of posaconazole (EC50 of 44.5 nM) or clotrimazole (EC50 of 200 nM) (Shing et al., 2020). Furthermore, isavuconazole at 70 μM completely prevented excystation of viable Acanthamoeba cysts (Shing et al., 2020). Potency against both trophozoite and cyst makes isavuconazole a promising drug candidate to block the propagation of trophozoite–cyst cycling of Acanthamoeba in Acanthamoeba keratitis.

In the context of our drug discovery and drug repurposing efforts, we pursued elucidation of the drug-target interactions for inhibitors targeting Acanthamoeba castellanii CYP51 (AcCYP51). In the course of these studies, we have observed an unusual property of AcCYP51 to form a stable dimer that sustained size-exclusion chromatography during purification. As we discovered subsequently, the dimerization occurred via a domain-swap mechanism through the exchange of the N-terminal regions between two protomers. Domain swapping has not been reported in CYP protein family P450 previously. In this article, we use abbreviation CYP to denote a P450 protein family, while P450 term is reserved for the ferrous CO-bound form with iron Soret band at ∼450 nm in order to distinguish it from the P420 form. The recombinant CYP enzymes of bacterial origin are monomeric, whereas CYP enzymes of higher eukaryotes, when expressed heterologously, tend to form random aggregates in solution, and their multimolecular assemblies have been detected in crystal structures (Reed and Backes, 2017).

At physiologic conditions, endoplasmic reticulum (ER)-bound CYP enzymes presumably exist as homo- or even hetero-oligomers. The fluorescence resonance energy transfer and bimolecular fluorescence complementation in living cells suggest that CYP2C2 forms homo-oligomers and that the homo-oligomerization is dependent on the signal membrane anchor sequence (Śzcześnaja-Korupa et al., 2003; Ozalp et al., 2005). Homodimerization in intact cellular membranes was suggested for the steroidogenic CYP17 and CYP19 by fluorescence resonance energy transfer coupled with quartz crystal microbalance and atomic force microscopy (Praporshik et al., 2009). Homodimerization for the drug-metabolizing CYP2C8 was demonstrated by cysteine-scanning mutagenesis and crosslinking of sulphydryl groups (Hu et al., 2010). Finally, homo- and hetero-oligomerization in microsomal membranes was demonstrated for CYP3A4, CYP3A5, and CYP2E1 by luminescence resonance energy transfer (Davydov et al., 2015). Despite these biophysical and biochemical observations, the CYP oligomerization mode is unknown. The random intermolecular protein-protein interfaces observed crystallographically are heterogeneous and planar and have a relatively small interaction area ranging from 290 to 550 Å² (Scott et al., 2003; Schoch et al., 2004; Ouellet et al., 2008; Reed and Backes, 2017).

In this work, we have structurally characterized CYP51 from the lower eukaryote A. castellanii strain Neff (AcCYP51), which is expressed with a truncated transmembrane helix. We found that only dimeric AcCYP51 had spectral characteristics typical of the functionally competent CYP enzymes. By X-ray crystallography, we demonstrated that AcCYP51 alone is dimerized via N-termini swapping, resulting in formation of a 2000 Å² nonplanar protein-protein interface. When bound to the azole inhibitors clotrimazole and isavuconazole, AcCYP51 crystallized in the monomeric form with the 74 N-terminal residues disordered in the AcCYP51-isavuconazole complex. The AcCYP51 X-ray structures confirmed a novel dimerization mechanism and elucidated differences in the clotrimazole- and isavuconazole-binding modes that plausibly explain the superior potency of isavuconazole against A. castellanii (Shing et al., 2020).

Materials and Methods

AcCYP51 Expression and Purification

AcCYP51, which is codon-optimized for bacterial expression, had a coding sequence with 42 N-terminal membrane-anchoring residues replaced with the MAKKTSSRGK. A hexahistidine tag was added at the C terminus to increase protein yield and recovery during purification (see Supplemental Data 1). This construct was generated synthetically (GenScript, Piscataway, NJ) and cloned into the pCW-LIC expression vector obtained from the nonprofit plasmid repository (Addgene, Cambridge, MA).

To improve the P450/P420 ratio, the original protocol used to isolate recombinant N. fowleri CYP51 (Dehnath et al., 2017) was modified (see Supplemental Data 2). Briefly, the modifications included a switch to the HMS174 Escherichia coli strain, coexpression of chaperones, omitting detergent 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) used initially to facilitate protein release from the membrane, and replacement of imidazole with histidine during elution from the Ni-NTA column. Exposure to imidazole led to quick loss of the 450-nm band in the absorbance spectra of the CO-bound ferrous AcCYP51. Finally, additional purification step of size-exclusion chromatography (SEC) on a Superdex 200 XK 26 column coupled to MALs (SEC-MALS) was used to separate Pf450 form, which was eluted as a dimer, from Pf420 form, which was eluted as a monomer. Collectively, after four chromatographic steps, including Ni-NTA affinity chromatography, Q-Sepharose ion-exchange chromatography, hydroxyapatite chromatography, and size-exclusion chromatography, fractions containing AcCYP51 with ~80% Pf450 content were pooled, concentrated to ~1 mM, aliquoted, and frozen at ~80°C.

SEC-MALS

SEC-MALS experiments were performed using in-line multianalyte light scattering (MALS) detector (miniDAWN; Wyatt Technology, Santa Barbara, CA) at 7°C. Two-milliliter protein sample was injected onto pre-equilibrated Superdex 200 XK26 column using 2-ml sample loop at a flow rate of 0.5 ml/min. The composition of the equilibration and sample buffer used was 50 mM potassium phosphate, pH 8.0, and 5% glycerol. Data of SEC-MALS thus obtained were analyzed by ASTRA 6.1 software provided by the instrument manufacturer.

UV-Visible Analysis of AcCYP51

The spectra were recorded using a Thermo Scientific Multispec Go UV-visible spectrophotometer. Protein samples were diluted in assay...
buffer (50 mM potassium phosphate, pH 8.0, and 10% glycerol) and allowed to equilibrate to room temperature for 10 minutes prior to readings. Spectra were recorded from 250 to 700 nm for the ferric and dithionite-reduced ferrous AcCYP51 in assay cuvette. Baseline was established using buffer alone and was subtracted from the sample signal prior to analysis using SkanIt software provided by the manufacturer. The CO difference spectrum was recorded by placing dithionite-reduced ferrous AcCYP51 into the sample cuvettes and recording the baseline. Then CO was bubbled into the same cuvette, and the difference spectrum was recorded. The concentration of AcCYP51 was approximated from the absorption peak at 450 nm using the extinction coefficient \( \varepsilon_{450} = 91 \text{m}^{-1} \text{cm}^{-1} \) (Omura and Sato, 1964).

To assess the spectral properties of the crystallized AcCYP51 dimer, crystals were harvested individually; each crystal was washed thoroughly in a well solution and then dissolved in 50 \( \mu \text{L} \) of assay buffer. The number of crystals required for a single analysis varied from 10 to 20 depending on the crystal size. The UV-visible spectra of the dissolved crystals were recorded at ambient temperature in 50-\( \mu \text{L} \) cuvette (952010077; Eppendorf). To generate ferrous-CO spectra, a few crystals of sodium dithionite were added to the CO-bubbled ferric protein sample.

### Inhibitor Binding by UV-Visible Spectroscopy

#### Type I and Type II Binding.

To determine binding modes of different ligands used in the study, 20-\( \mu \text{M} \) ligand stock solutions were prepared in corresponding solvents. Ivasuconazole and clotrimazole were prepared in DMSO, and 31-norlenosterol was dissolved in isopropanol. Prior to analysis, 5 \( \mu \text{M} \) AcCYP51 in assay buffer was mixed with 20 \( \mu \text{M} \) ligand. After 30 minutes of incubation at room temperature, absorbance spectra were recorded from 300 to 500 nm. To determine difference spectra, blank readings were taken for protein alone in assay buffer with respective ligand vehicle under given experimental conditions.

#### Binding Kinetics.

To determine binding kinetics of the ligands, 5 \( \mu \text{M} \) AcCYP51 in assay buffer was mixed with 20 \( \mu \text{M} \) ligand. After mixing, spectra from 300 to 500 nm were recorded every 5 minutes of incubation. Blank readings were determined from incubation of protein alone with respective ligand vehicle under given experimental conditions.

AcCYP51 stock concentration was determined by absorbance of the CO-bound ferrous form at 450 nm \( \varepsilon_{450} = 91 \text{m}^{-1} \text{cm}^{-1} \) (Omura and Sato, 1964). Concentration of the AcCYP51-norlanosterol complex was determined using the peak-to-trough extinction coefficient, \( \varepsilon_{390-420} = 100 \text{m}^{-1} \text{cm}^{-1} \) (Luthra et al., 2011). Concentration of the AcCYP51-inhibitor complex was estimated using the peak-to-trough extinction coefficient, \( \varepsilon_{138-411} = 110 \text{m}^{-1} \text{cm}^{-1} \) (Wang et al., 2012).

#### Binding Isotherms.

The DMSO stock solutions for clotrimazole and isavuconazole were freshly prepared at concentrations of 0.2 and 0.4 mM. The AcCYP51 stock was diluted to 1 \( \mu \text{M} \) in assay buffer. Two milliliters of the AcCYP51 solution was split evenly into a reference and a sample cuvette (1-cm polymethyl methacrylate cuvette (cat. no. 759150; BrandTech Scientific, Essex, CT)). The AcCYP51 solution was allowed to equilibrate for 30 minutes to room temperature prior to absorption readings. The absorption readings were performed at 20°C on a Cary 1E Dual Beam UV-visible spectrophotometer (Varian). The experiment was conducted with two replicates.

In the course of titration, 1 \( \mu \text{L} \) of DMSO was added to the reference cuvette, whereas 1 \( \mu \text{L} \) of inhibitor dissolved in DMSO was added to the sample cuvette in 200-\( \mu \text{L} \) (data points 1–4) and 400-\( \mu \text{L} \) (data points 5–10) increments. The cuvette content was mixed with a transfer pipette prior to each reading. Absorbance readings were taken from 350 to 500 nm, and the binding isotherm was generated by plotting the differences between the absorbance minimum at 410 nm and absorbance maximum at 430 nm as a function of added drug concentration.

The data were analyzed in GraphPad Prism 6.07 with the rearrangement of the Morrison binding equation (Morrison, 1969) to determine the dissociation constants:

\[
\Delta A = \left( \frac{\Delta A_{\text{max}}}{2[|E|]} \right) \left( K_D + [L] \right) + [E] \cdot \left( K_D + [E] + [L] \right)^2 - 4[E][L]^{0.5}.
\]

in which \( \Delta A \) is the difference between absorbance maximum and minimum, \( \Delta A_{\text{max}} \) is the extrapolated maximum absorbance difference, [L] is the ligand concentration, and [E] is the enzyme concentration.

### Crystallization and Structure Determination

Prior to crystallization, AcCYP51 stored at -80°C in 50 mM potassium phosphate, pH 8.0, and 5% glycerol was diluted 2-fold to 0.5 mM with water or buffer containing a ligand at 1.2 molar access. Screened of crystallization conditions was performed using commercial high-throughput screening kits available in deep-well format from Hampton Research (Aliso Viejo, CA) or Qiagen (Germantown, MD), a nanoliter drop-setting Mosquito robot (TTP LabTech, Melbourne, UK) operating with 96-well plates, and a hanging drop crystallization protocol. For diffraction quality, crystals were further optimized in 96-well plates configured using the Dragonfly robot (TTP LabTech) and the Designer software (TTP LabTech). All crystals were obtained at 23°C. Clotrimazole and isavuconazole stock solutions were prepared fresh in DMSO. The 1:1.2 molar ratio protein-inhibitor mix was incubated for 30 minutes on ice prior to mixing with the well solutions. Optimized crystallization conditions are provided in Table 1.

Diffraction data were collected remotely at beamline 8.3.1, Advanced Light Source, Lawrence Berkeley National Laboratory. Data indexing, integration, and scaling were conducted using XDS (Kabsch, 2010). T. cruzi CYP51 structure [sequence identity 38%, Protein Data Bank (PDB): 4C27] was used as a molecular replacement model. The initial AcCYP51 model was built and refined using the BUCANNEER and REFMAC5 modules of the CCP4 software suite (Collaborative Computational Project, Number 4, 1994) and COOT software (Emsley and Cowtan, 2004). Data collection and refinement statistics are shown in Table 1.

### Molecular Modeling and Simulation

A full-length AcCYP51 homodimer was constructed computationally by modeling the transmembrane (TM) helix and its flanking regions into the AcCYP51 structure using the Rosetta MP package v3.0 (Koehler Leman et al., 2017). The TM helix (residues 10–30) was modeled ab initio using the helix_from_sequence program (Koehler Leman et al., 2017). The TM helices of each monomer were positioned diagonally opposite one another in agreement with the position of the N termini in the crystal structure. The flexible linker region (residues 31–52) and the N terminus were built for each protomer using the mp_domain_assembly program (Koehler Leman and Bonneau, 2018). Five hundred models were generated, and the structure with the lowest value of the Rosetta energy function was selected for further analysis. The energy minimization of the full-length dimer was performed to optimize interatomic distances and angles. Then, the 50-nanosecond molecular dynamics (MD) simulations were conducted to refine the structure of the linker regions and to obtain the proper arrangement of them in respect to the rest of the dimer. In the course of the simulation, the flexible linkers and the N termini were allowed to move freely, whereas harmonic restraints were applied to the backbone atoms of the rest of the protein.

The fully assembled AcCYP51 dimer was embedded into a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphothanolamine (POPE)/cholesterol lipid bilayer composed of 75% POPC, 18% POPE, and 6% cholesterol, corresponding to the abundance of these lipids in the ER (Srejber et al., 2018). Cholesterol is the closest analog of ergosterol and ergosterol-like lipids in A. castellanii membranes available in Amber data base and widely used in Amber force-field simulations. The protein-membrane model
was constructed using CHARMM-GUI (Jo et al., 2008). The simulation was conducted in a box containing protein, lipid membrane, water molecules, and ions. The AMBER14SB (Maier et al., 2015) and LIPID17 (Case et al., 2018) force fields were used for protein and lipids, respectively, whereas the heme group parameters were taken from Rydberg et al. (2007). Protonation states of the amino acid residues were determined at physiologic pH (pH = 7.14) using the PDB2PQR server (Dolinsky et al., 2004). Systems setup was performed with tleap program of Amber18 (Case et al., 2018). The system was solvated with explicit transferable intermolecular potential with 3 points (TIP3P) water molecules (Jorgensen and Jenson, 1998) in a cubic box extending at least 10 Å from the solute surface treated with periodic boundary conditions. Net charges were neutralized by replacing water molecules with Na+ and Cl\(^-\) ions. All MD simulations were conducted using NAMD v.2.13 program (Phillips et al., 2005). The 50,000 steps of energy minimization were performed to eliminate the atomic clashes. The lipid bilayer equilibration procedure was performed at constant pressure (1 atm) and constant temperature (298 K), for 150 nanoseconds with 1 kcal\(\text{mol}^{-1}\)Å\(^2\) harmonic position restraints applied to the protein backbone and heme. Further equilibration of the systems was performed at 1 atm and 298 K with a constant ratio constraint applied to the lipid bilayer in the X-Y plane.

### Results

**Oligomerization of AcCYP51 in Solution.** An expression construct of AcCYP51 lacking the transmembrane helix and containing an exogenous 10–amino acid lead sequence at the N terminus and a hexahistidine tag at the C terminus was synthesized (Supplemental Data 1). Four chromatographic steps were used to purify AcCYP51: affinity chromatography on Ni-NTA resin, ion-exchange chromatography on Q Sepharose, hydroxyapatite chromatography, and, finally, size-exclusion chromatography on Superdex 200 XK 26 coupled to multi-angle light scattering (SEC-MALS).

### Table 1

<table>
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<th>Oligomerization</th>
<th>Clotrimazole (CL6)</th>
<th>Isavuconazole (QKM)</th>
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<td>P1</td>
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<td>6</td>
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<td>83,269 (6049)</td>
<td>74,605 (5556)</td>
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<td>19.3 (342.7)</td>
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<td>Ivo(^b)</td>
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<td>7.1 (0.6)</td>
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<td>Outliers (%)</td>
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\(^a\)Asymmetric unit
\(^b\)Data for the highest resolution shell are shown in parentheses.
\(^c\)Polyethylene glycol
\(^d\)Temperature factor
\(^e\)Not applicable
\(^f\)Root-mean-square
of the P1 fraction determined by MALS constituted 100.0 ± 9.5 kDa (Fig. 1C). On SDS-PAGE, P1 migrated consistent with the MM calculated for one polypeptide chain (52.4 kDa) (Fig. 1D). These data demonstrated that 1) AcCYP51 forms a dimer in solution and 2) the dimer is stable enough to undergo size-exclusion chromatography. We have not observed any higher oligomers, suggesting that the highest oligomeric state of AcCYP51 in solution was a dimer.

UV-Visible Spectroscopic Properties of Recombinant AcCYP51. The dimeric and monomeric protein fractions were separated by size-exclusion chromatography and were individually analyzed by UV-visible spectroscopy (Fig. 2). The ferric AcCYP51 dimer, P1 fraction, had a typical CYP spectrum with the absorbance maxima at 418 nm and the ratio of absorbance at 2.9 Å. In both structures, the asymmetric unit contained six crystallographically resolved P450 enzymes (Lewis et al., 2006). Overall X-Ray Structures of the Inhibitor-Bound AcCYP51. AcCYP51 co.crystallized with clotrimazole or isavuconazole as a monomer; crystals diffracted to a resolution of 2.9 Å. In both structures, the asymmetric unit contained six inhibitor-bound AcCYP51 molecules. In each molecule, electron density for the bound inhibitor was unambiguously defined as evidenced by the omit maps in Fig. 4, A and B. In clotrimazole-bound AcCYP51, the β1-1 region folded back to its own polypeptide chain, restoring a typical P450 scaffold. Two consecutive glycine residues in the A-A1-1 strand and the A-A1-2 of the interacting protomer instead of folding with the own polypeptide chain (Fig. 3A). Because of the swap, the main chain of the K40-G41-K42 fragment participates in intermolecular protein-protein interactions, whereas the lysine side chains face the bulk solvent.

The overall protein scaffold was similar to that of monomeric CYP enzymes with the qualification that the region of K40-P52 containing the first strand of the β-sheet-1 (β1-1) was swapped between the two protomers and was parallel to the β1-2 of the interacting protomer instead of folding with the own polypeptide chain (Fig. 3A). A dimer protein-protein interface of 2000 Å² was calculated using the Protein Interfaces, Surfaces, and Assemblies software (Krissinel and Henrick, 2007). Altogether, 16 H-bonds and two salt bridges stabilized the protein-protein interface formed by 40 hydrophobic residues provided by each protomer. The buried surface constituted 10% of the accessible surface of AcCYP51 and provided total solvation energy gain of −35.4 kcal/mol. F84 had the most prominent single residue input of −2.1 kcal/mol (Fig. 3C). This protein-protein interface was strong enough to sustain size-exclusion chromatography and crystallization in up to 1 M urea. Crystals obtained in 0.55 M urea diffracted to 1.85 Å and had intact dimeric structure similar to that seen in native conditions.

Heme Binding. An unusual feature of the AcCYP51 dimer was heme “wobbling.” Despite the multiple H-bonding interactions with Y114, Y127, and Q110 and a salt bridge with R368 formed by the heme propionate moieties, two alternative conformations were required to approximate the heme position in the ligand-free AcCYP51 (Fig. 3D). In heme conformers, the Fe-S bond length was refined between 2.33 and 2.41 Å. The 2.41-Å bond length goes beyond the range reported for other crystallographically resolved P450 enzymes (Lewis et al., 2006). Consistent with the ferric resting state, the water molecule was modeled in both protomers as a sixth, axial iron ligand at the distances of ≈2.64 Å of the heme iron (Fig. 3D).

Overall X-Ray Structure of the AcCYP51 Dimer. The dimeric AcCYP51 readily crystallized in the absence of added ligands. The crystal structure at 1.8 Å revealed a symmetrical head-to-head homodimer with swapped N termini (Fig. 3A). The first seven amino acids of the lead sequence were disordered and not visible in electron density. The next three exogenous residues from the lead sequence (K40-G41-K42, yellow in Fig. 3B) followed by authentic AcCYP51 sequence participated in the N-terminal swapping. As shown in Fig. 3B, only the main chain of the K40-G41-K42 fragment participates in intermolecular protein-protein interactions, whereas the lysine side chains face the bulk solvent.

Crystals obtained in 0.55 M urea diffracted to 1.85 Å and had intact dimeric structure similar to that seen in native conditions.
AcCYP51-isavuconazole structure, the β1-1, A', and A atomic coordinates are not included.

AcCYP51-Clotrimazole Complex in the Crystal. The structure determined in this work is the first of the CYP51-clotrimazole complex. Similar to other P450-clotrimazole structures [CYP46A1 (PDB ID: 3MDV), P450 BM3 (6H1T), EryK (2XFH), and OleP (4XE3)], clotrimazole bound in the active site of AcCYP51 via a coordination bond provided to the heme iron by the aromatic nitrogen of the imidazole moiety and via the hydrophobic interactions mediated by the phenyl moieties of the drug. The orientation of the chlorophenyl moiety in clotrimazole varies between different CYP enzymes. In AcCYP51, the short side chain of S117 defines orientation of the chlorophenyl moiety by providing space to accommodate a bulky Cl substituent that is within 5.1 Å of the serine carboxyl group (Fig. 4A). Other contacts within 5 Å of clotrimazole involve Y114, F116, S117, F121, V126, T127, L216, A290, F293, A294, H297, L363, and V366. Compared with the inhibitor-free AcCYP51, the first-tier residues in the substrate-binding site are shifted away from clotrimazole to accommodate the inhibitor. To compensate for the inhibitor-introduced distortions, this trend was propagated to the second and third tier residues. From the perspective of drug design, the tight fit in the active site leaves room for derivatization of only one phenyl moiety in clotrimazole.

AcCYP51-Isavuconazole Complex in the Crystal. The structure determined in this work is the first of the P450-isavuconazole complex. Similar to clotrimazole, isavuconazole bound in the heme pocket via coordination to the heme iron and protein-drug interactions (Fig. 4B). In the heme pocket, the set of interacting residues is similar to that of clotrimazole excluding L216 and H297. More-elongated isavuconazole molecule also makes interactions with F365, M367, and M471 with the thiazolyl benzonitrile moiety of the drug. The nitrile group points toward the opening created by disordering of the A' and F' helices.

Stability of the AcCYP51-Ligand Complexes in Solution. The binding of the inhibitors and substrates was assessed by the shift of the Fe Soret band in the UV-visible spectra of dimeric AcCYP51 (Fig. 5). Type II binding spectra were obtained upon clotrimazole and isavuconazole binding (Fig. 5A). Type I spectra were obtained upon binding sterols lanosterol and 31-norlanosterol, with qualification that 31-norlanosterol generated a larger spectral response than lanosterol (Fig. 5B). Binding kinetics of AcCYP51 at saturating ligand concentrations showed that both isavuconazole and clotrimazole reached saturation for 10–15 minutes, whereas more than 30 minutes were required for 31-norlanosterol to reach the saturation (Fig. 5, C and D). Magnitude of the spectral changes suggested that only 10%–20% of AcCYP51 resulted in the formation of enzyme-ligand complexes (Table 2). When AcCYP51 was titrated with clotrimazole, a typical binding curve was obtained, and $K_D$ of 152.3 ± 10.0 nM was calculated by fitting binding data using the Morrison “quadratic” equation (Morrison, 1969) (Fig. 5E). For isavuconazole, the binding plateau could not be reached (Fig. 5E). In the context of our structural data, isavuconazole may have affected integrity of AcCYP51 and the magnitude of the Soret spectral shift by partial protein unfolding.

Modeling AcCYP51 Dimer Interactions with the ER Membrane. To assess compatibility of the domain-swap dimerization with membrane binding, we built a molecular
model of AcCYP51-membrane interactions. The wild-type N-terminal residues omitted from the recombinant AcCYP51 were added computationally. The fully reconstructed AcCYP51 dimer was embedded into a lipid membrane constituted of phospholipids, POPC/POPE, and cholesterol found in the membranes of higher eukaryotes. Cholesterol was used in MD simulations to optimize conformation of the TM helix (residues 10–30) and its flanking regions because the Amber force field does not have the parameter for ergosterol found in the membranes of lower eukaryotes.

The model demonstrated that the dimerization mode observed in the crystal structure is compatible with the membrane association (Fig. 6A). The TM helices are separated in space and run virtually orthogonal to the lipid bilayer, which is consistent with the relatively short TM helix in AcCYP51. The region connecting the TM helix with the globular CYP domain is predicted to be a flexible loop up to the downstream segment 44–51, which adopts a \( \beta \)-strand structure (\( \beta \)-1-1) running parallel to \( \beta \)-1-2 of the interacting protomer.

In addition to the TM helix, protein-lipid interactions occur through the regions corresponding to residues 31–43 (Fig. 6B). This fragment has several charged amino acids (i.e., K31, R33, E34, R36 and K37), which interact with the zwitterionic heads of POPC and POPE phospholipids. On the other hand, the presence of hydrophobic residues, such as V32 and L43, enables the nonpolar interactions between the linker regions and lipid tails (Fig. 6B). Finally, W35, Y38, and Y41 act as anchoring residues, which are usually located between the polar group and the hydrophobic core of the lipid bilayer in the glycerol region of the membrane (Mustafa et al., 2019).

The \( \beta \)-5-\( \beta \)-6 segment (residues 372–380) also faces the membrane in our model and is partially immersed in its hydrophobic environment. Finally, in one of the monomers, residues R273, G274, and E275 tend to associate with lipid heads (Fig. 6B). The electrostatic potential density at the surface of AcCYP51 dimer shows that the membrane-associated protein surface comprises charged patches, thus supporting the reliability of our membrane-insertion model (Fig. 6C).

**Discussion**

CYP enzymes are a superfamily of \( \beta \)-type heme-containing monoxygenases descended from a single common ancestor (Gotth, 2012). They share structural features, such as a common protein scaffold, the similarity in positioning of the heme group, and the access/egress pathways for substrates and products (Otyepka et al., 2007). The heme group is bound via a thiolate sulfur bond donated by the universally conserved “proximal” cysteine residue at the fifth, axial coordination of the heme iron. The heme iron binds molecular oxygen, \( \text{O}_2 \), as
a sixth ligand in the “distal” pocket, which also serves as a site for substrate binding. CO resembles O₂ in size and heme-binding properties. By replacing O₂, CO blocks the enzymatic turnover of the CYP enzymes. CO is used as a sensitive probe of local conformation and dynamics in the active site of heme-thiolate proteins. Absorption spectra of the CO-bound ferrous CYP enzymes (Fe⁺⁻CO) often display two Soret bands in the blue region of the visible range, which are denoted P₄₂₀ and P₄₅₀. The 420- and 450-nm bands are arguably assigned to CYP species having, respectively, a protonated (thiol) and deprotonated (thiolate) cysteine side chain as axial iron ligands (Perera et al., 2003; Dunford et al., 2007; Sabat et al., 2009; Driscoll et al., 2011). Alternatively, recruitment of a histidine residue to replace the native cysteine thiolate ligand has been suggested (Martinis et al., 1996; Sun et al., 2013). Finally, theoretical calculations indicate that stretching the Fe-S bond by only 0.2 Å could induce the spectral transition of ferrous CO P₄₅₀ to P₄₂₀ (Jung et al., 1979). A variety of extreme conditions, such as heating, hydrostatic pressure, organic solvents, and denaturants, were used to convert P₄₅₀ to P₄₂₀ (Martinis et al., 1996; Sun et al., 2010; Andse and Blackburn, 2018; Chen et al., 2019). On a few occasions, P₄₂₀ could be converted back to P₄₅₀ (Ogura et al., 2004; Dunford et al., 2007).

The mechanistic knowledge accumulated in the field supports the assumption that a CYP monomer is sufficient for catalytic function, and CYP dimerization is not required for the act of catalysis. At the same time, there are reports of CYP-CYP interactions both in microsomal membranes (Greinert et al., 1982; Kawato et al., 1982; Myasoedova and Berndt, 1990; Schwarz et al., 1990; Myasoedova and Magretova, 2001; Szczesna-Skorupa et al., 2003; Ozalp et al., 2005; Praporski et al., 2009; Hu et al., 2010; Davydov et al., 2015) and in recombinant CYP proteins (Myasoedova and Berndt, 1990; Von Wachenfeldt and Johnson, 1995; Von Wachenfeldt et al., 1997; Davydov et al., 2005, 2010, 2013; Reed et al., 2012). CYP-CYP interactions are suggested to play regulatory rather than catalytic role (Reed and Backes, 2017). Despite biophysical and biochemical evidence, details of CYP-CYP interaction mode(s) remain obscure. The protein-protein interfaces reported in the crystal structures of the multimeric complexes of eukaryotic CYP enzymes are random and have small interaction areas (Reed and Backes, 2017). These considerations encouraged us to determine the first X-ray structure of a sustainable CYP51 dimer. This in turn led to discovery of the domain-swap dimerization mode.

The P₄₅₀ character of the AcCYP51 dimer contrasts with the P₄₂₀ character of the AcCYP51 monomer. The propensity of AcCYP51 to convert into a P₄₂₀ form even in mild purification conditions was consistent with the flexibility of the heme pocket manifested by heme wobbling and lengthening of the Fe-S bond as observed in the crystal structure. We speculate that dimerization in AcCYP51 plays a stabilizing role to maintain the functional status of the heme.

The modifications introduced at the N terminus of AcCYP51 to enable expression in the bacterial host may potentially
Fig. 5. Ligand-binding properties of the AcCYP51 dimer. (A) Type II difference binding spectra of 20 μM isavuconazole (dashed line) and 20 μM clotrimazole (solid line) to 5 μM AcCYP51. (B) Type I difference binding spectra of 20 μM 31-norlanosterol to 5 μM AcCYP51. (C) Binding kinetics of 20 μM isavuconazole (open squares) and clotrimazole (open circles) to 5 μM AcCYP51 at 430 nm (red curve) and 411 nm (black curves). (D) Binding kinetics of 20 μM 31-norlanosterol to 5 μM AcCYP51 at 388 nm (red curve) and 418 nm (black curve). Experiments in (C and D) were performed twice. A representative time course is shown for each ligand. Percentage of ligand-bound fraction (10%–20%) and the monomeric form of the AcCYP51-isavuconazole and AcCYP51-clotrimazole complexes observed in the crystals. Partial denaturing of AcCYP51 in response to inhibitor binding (74 disordered N-terminal residues in the AcCYP51-isavuconazole complex) suggests a mechanism of action fundamentally different from conventional enzyme inhibition by blocking the active site. In living cells, structurally aberrant AcCYP51 may undergo further denaturation and be permanently deactivated by proteolytic degradation. In the context of the superior activity of isavuconazole, this

TABLE 2
UV-visible quantification of the AcCYP51 ligand-bound fraction

<table>
<thead>
<tr>
<th>Ligand</th>
<th>AcCYP51 ligand, μM</th>
<th>Fraction of ligand-bound AcCYP51, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n1</td>
<td>n2</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>20.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Isavuconazole</td>
<td>20.0</td>
<td>5.0</td>
</tr>
<tr>
<td>31-Norlanosterol</td>
<td>20.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*Experiments were performed in duplicates. Values for the replicates n1 and n2 are shown separately.
phenomenon can be exploited for designing other AcCYP51 inhibitors that target the dimerization interface.

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Authorship Contributions

Participated in research design: Sharma, Hernandez-Alvarez, Podust.
Conducted experiments: Sharma, Shing.
Contributed new reagents or analytic tools: Hernandez-Alvarez.
Performed data analysis: Sharma, Shing, Debnath, Podust.
Wrote or contributed to the writing of the manuscript: Sharma, Hernandez-Alvarez, Podust.

References
