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Spin equilibrium and O₂-binding kinetics of *Mycobacterium tuberculosis* CYP51 with mutations in the histidine–threonine dyad

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Abstract

The acidic residues of the “acid–alcohol pair” in CYP51 enzymes are uniformly replaced with histidine. Herein, we adopt the *Mycobacterium tuberculosis* (mt) enzyme as a model system to investigate these residues’ roles in finely tuning the heme conformation, iron spin state, and formation and decay of the oxyferrous enzyme. Properties of the mtCYP51 and the T260A, T260V, and H259A mutants were interrogated using UV–Vis and resonance Raman spectroscopies. Evidence supports that these mutations induce comprehensive changes in the heme environment. The heme iron spin states are differentially sensitive to the binding of the substrate, dihydrolanosterol (DHL). DHL and clotrimazole perturb the local environments of the heme vinyl and propionate substituents. Molecular dynamics (MD) simulations of the DHL–enzyme complexes support that the observed perturbations are attributable to changes in the DHL binding mode. Furthermore, the rates of the oxyferrous formation were measured using stopped-flow methods. These studies demonstrate that both HT mutations and DHL modulate the rates of oxyferrous formation. Paradoxically, the binding rate to the H259A mutant–DHL complex was approximately four-fold that of mtCYP51, a phenomenon that is predicted to result from the creation of an additional diffusion channel from loss of the H259–E173 ion pair in the mutant. Oxyferrous enzyme auto-oxidation rates were relatively constant, with the exception of the T260V–DHL complex. MD simulations lead us to speculate that this behavior may be attributed to the distortion of the heme macrocycle by the substrate.

Keywords

CYP51; Cytochrome P450; Stopped-flow spectroscopy; Molecular dynamics; Resonance Raman spectroscopy

1. Introduction

The cytochromes P450 are among the most flexible enzymatic catalysts using O₂ and reducing equivalents from nicotinamide cofactors to afford a breadth of oxidative transformations [1–5]. Entry into the catalytic cycle occurs with a substrate binding event that often, though not always displaces a water molecule from the hexacoordinate ferric heme center, inducing a low- ($S = 1/2$) to high- ($S = 5/2$) spin transition. The corresponding increase in the redox potential facilitates reduction to the ferrous state and subsequent O₂ binding. The resulting oxyferrous intermediate undergoes reduction to a ferric peroxo species that, in turn, is dually protonated by a putative hydrogen-bonded network. Protonation cleaves the O–O bond to afford an oxoferryl π -cation radical, also known as Compound I, that is generally accepted to be the hydroxylating species in canonical cytochrome P450 catalysis [6,7]. Two amino acids, typically aspartate and threonine, are broadly conserved among these enzymes and have been established as critical components of the proton delivery system [8–21]. However, this axiom is largely based on extensive experimental and theoretical studies of cytochrome P450cam (CYP101). By contrast, residues replacing the “acid–alcohol” pair have been identified in other cytochrome P450 enzymes, although their roles in catalysis remained undefined [22].

A distinguishing feature of one P450 family, the sterol 14 α -demethylases (CYP51), is that the acidic residues of the acid–alcohol pair are uniformly replaced with histidine [23]. The active sites of mtCYP51 [24] and CYP101 including the relevant regions of the I-helix are illustrated in Fig. 1. This superimposition highlights the structural equivalence of the HT dyad and acid–alcohol pair. Summarized in Scheme 1, CYP51s demethylate 14 α -methylated steroids such as dihydrolanosterol (DHL) [25]. This transformation requires three revolutions of the P450 catalytic cycle, each requiring O₂ and two electrons. The first two steps are apparently Compound I-mediated hydroxylations that yield a 14 α -carboxaldehyde. These are followed by a mechanistically enigmatic third step that removes the carboxaldehyde as formate with concomitant loss of the 14 α -hydrogen. Biochemical experiments utilizing isotopically labeled substrates have concluded that the ferric peroxo intermediate mediates the third step, but the precise mechanism remains elusive [26]. To catalyze these two mechanistically distinct reactions, the enzyme must switch between the effective use of Compound I and peroxo intermediates. This catalytic strategy is generally considered to require reorganization of the proton delivery machinery prior to the third step. Molecular dynamics (MD) simulations and hybrid quantum mechanics/molecular mechanics studies in our group support this notion [27,28]. While these results confirm the value of theory to distinguish physically permissible mechanisms from unrealistic ones, in this case complementary experiments are necessary to confirm whether redundant proton-transfer pathways are operative in CYP51 catalysis.

To attain a comprehensive understanding of CYP51 catalysis, a complete picture of the catalytic cycle including the mechanisms of oxygen binding, electron and proton transfer, and identification of the elusive intermediates in the deformylation step are of substantial importance. The first reactive oxygen intermediate and bifurcation point of the canonical catalytic cycle is the oxyferrous species, which may proceed to productive oxidation of the substrate or auto-oxidize to yield the ferric enzyme and superoxide anion. Like the acid–

alcohol pair in other P450 enzymes, the HT dyad of the CYP51 family may similarly tune the spin state of the heme iron, modulate the rate of oxygen binding, and stabilize the resulting oxyferrous complex from auto-oxidation [29–32]. In this study, we adopt the *M. tuberculosis* (mt) enzyme as a model system and take the first steps toward dissecting the relationships between conserved active site residues and enzyme mechanism. UV–Visible (UV–Vis) absorption and resonance Raman (rR) spectroscopy were used to probe both the heme environment and electronic structure of the ferric enzyme in response to ligand binding. Furthermore, the impact of mutations and substrate on the rates of O₂ binding to the ferrous enzymes and subsequent auto-oxidation of the oxyferrous species are investigated. Changes in these parameters are interpreted in light of molecular dynamics simulations and computational predictions of O₂ migration pathways into the active sites.

2. Materials and methods

2.1. Synthesis of 24,25-dihydrolanosterol and cyclodextrin encapsulation

Lanosterol (55–65%; Steraloids, Newport, RI) was purified by silica gel chromatography using ethyl acetate:hexane (1:4) as a solvent. To lanosterol (2 mmol, 0.85 g) in dichloromethane (40 mL) 5% Pd/C [20% (w/w), 0.17 g] was added and the mixture hydrogenated at room temperature at 50 psi for 24 h. The resulting mixture was filtered through celite, and the filter cake was washed thrice with 5 mL of dichloromethane. The solvent was evaporated to afford 24,25-dihydrolanosterol (DHL) as a white solid in quantitative yield. To improve the aqueous solubility, DHL was encapsulated in 2-hydroxypropyl- β -D-cyclodextrin (HPCD). To 9.5 mL of a stirring and refluxing solution of 45% (w/v) HPCD in 100 mM potassium phosphate buffer (pH 7.5), 2.1 mg of DHL was added and allowed to dissolve. After cooling to room temperature, the solution was diluted to 500 μ M DHL. HPCD-encapsulated DHL (HPCD-DHL; DHL hereafter) was aliquoted and lyophilized for storage and reconstituted with deionized water prior to use.

2.2. Construction of mtCYP51 mutants

The mtCYP51-pET-17b expression plasmid was a gift from Larissa Podust (University of California-San Francisco) [25]. The *CYP51* mutants were constructed using the Quikchange (Stratagene) mutagenesis method where DpnI (New England Biolabs) was used to digest the parental DNA. Transformation of the remaining DNA resulted in colonies that were screened for the desired mutation by DNA sequencing.

2.3. Protein expression and purification

mtCYP51 and its mutants were over expressed and isolated from HMS174 (DE3) *Escherichia coli* cells. A single colony was used to inoculate 100 mL of LB media with ampicillin (100 μ g/mL) and grown overnight at 37 °C with shaking at 200 rpm. The overnight culture (15 mL) was used to inoculate 1 L of LB media containing ampicillin (100 μ g/mL), potassium phosphate (100 mM, pH 7.4) and glycerol (5% v/v). The expression culture was incubated at 37 °C with shaking at 180 rpm. When the A₆₀₀ reached 0.4 to 0.6, the temperature was decreased to 20 °C (18 °C in the case of the T260V mutant) and the shaking to 150 rpm. When the A₆₀₀ reached 0.6 to 0.8, the culture was induced with 0.2 mM oxoferryl π -cation radical and 0.5 mM δ -aminolevulinic acid. After 20 h of growth, the cells

were harvested by centrifugation at $5000 \times g$ and 4°C for 10 min. Cell pellets were resuspended in lysis buffer (3 mL/g cell pellet) containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% (v/v) glycerol, and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was lysed by two passes through a high-pressure homogenizer at 25000 psi. The lysate was centrifuged at $10,000 \times g$ for 45 min. The supernatant was loaded onto Ni^{2+} -NTA agarose resin (Qiagen) that had been previously equilibrated with the lysis buffer. The column was washed with 1.5–2 L of the lysis buffer and proteins were then eluted with the same buffer containing 50 mM imidazole. Red-colored fractions were pooled and concentrated with Amicon filtration units (Millipore) with a molecular weight cutoff of 30 kDa. Concentrated protein was loaded onto a Superdex-75 column previously equilibrated with 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 5% (v/v) glycerol. Proteins were eluted with the same buffer at a flow rate of 0.2 mL/min. Red fractions with a A_{419}/A_{280} ratio of 1.4 or greater were pooled and the purity of these preparations were confirmed by SDS-PAGE. CO binding assay was performed by monitoring the absorbance at 450 nm after complete reduction with sodium dithionite and bubbling with CO. Spectra of carbonmonoxyferrous mtCYP51 and mutants consistently have Soret, α , and β bands at 449 nm, 542 nm, and 574 nm, respectively. Cyto-chrome P450cam was expressed and purified as previously described [33]. Proteins were stored at -180°C until required.

2.4. UV-Vis spectroscopy ligand binding titrations

Binding of DHL and clotrimazole (CLO) to mtCYP51 and mutants were monitored in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl and 5% (v/v) glycerol using an Olis Cary 14 Conversion double-beam UV-Vis spectrometer. After recording a baseline with ferric protein, CLO dissolved in ethanol or DHL aqueous solution was incrementally added to the sample cell, while the corresponding volume of solvent was added to the reference cell. The total volume of additional solvent did not exceed 2% (v/v). The difference in absorbance between the wavelength minima and maxima (ΔA) were plotted against the inverse of the DHL or CLO concentrations and the K_d values were determined by a linear regression fit to the equation $1/\Delta A = (K_d/\Delta A_{\text{max}})(1/[L]) + 1/\Delta A_{\text{max}}$. The percent of ferric enzymes in the high-spin state (%HS) were computed using the relative absorbances at 646 nm (A_{646}) and 419 nm (A_{419}) as well as the corresponding extinction coefficients for the low-spin (ϵ_{419}) and high-spin components (ϵ_{419}) using the equation $\%HS = 100A_{646}/((\epsilon_{646}/\epsilon_{419})A_{419} + A_{419})$ [32,34].

2.5. Resonance Raman spectroscopy

RR spectra were obtained following excitation using the 406.7 nm line of a Coherent Innova 302C krypton ion laser. Laser powers at the sample were 35 mW. Spectra were collected using a f/9.7 single grating monochromator (Acton SP2750, Princeton Instruments) at a 15 micron slit width using 1800 grooves/mm gratings, and imaged using a 1340×400 pixel back-illuminated CCD camera with UV optimized coatings (PyLoN 400BR eXcelon, Princeton Instruments). Reference calibrations were performed with respect to a Hg vapor lamp. The nonlinear fluorescence background of resonance Raman spectra were removed using asymmetric least squares smoothing [35] in MATLAB.

2.6. Stopped-flow UV–Vis spectroscopy kinetic studies

Buffers used in the stopped-flow kinetic experiments were thoroughly degassed using multiple rounds of a freeze–pump–thaw procedure in Schlenk tubes attached to a gas/vacuum manifold. In brief, buffer-filled Schlenk tubes were submerged in liquid N₂ until completely frozen. The remaining headspace was thoroughly evacuated by opening it to vacuum and then closed. The buffer was allowed to fully thaw and the procedure was repeated with at least three additional cycles. When the degassing procedure was complete, the buffers were stored under an atmosphere of N₂. mtCYP51 proteins in 50 mM Tris–HCl (pH 8.0) buffer were purged with N₂ on a sealed gas manifold for 20 min at 4 °C and transferred to an N₂-filled glovebox. The proteins were reduced by the addition a few crystals of sodium dithionite and complete reduction were monitored by UV–Vis absorption spectroscopy. Excess dithionite was removed using a Sephadex G-25 HiTrap desalting column previously equilibrated with degassed buffer. Degassed buffer was oxygenated by bubbling O₂ for 30 min. Dissolved O₂ concentrations were determined using the azide modification of the Winkler method [36].

Kinetic data were acquired using an Applied Photophysics SX-18MV stopped flow UV–Vis spectrophotometer equipped with a xenon lamp and photodiode array detector. The dead time of the instrument was determined to be 1.2 ms using the method described by Matsumura et al. [37]. After flushing with degassed buffer, the two syringes were respectively loaded with oxygenated buffer and ferrous mtCYP51 or mutants in anaerobic buffer. Concentrations provided in the Figures are those after mixing in the 350 µL flow cell. The photodiode array collected 800 spectral scans over 2 s. The kinetic data sets were fit to the reaction model $Fe^{2+} \xrightarrow{k_1} Fe^{3+}O_2^- \xrightarrow{k_2} Fe^{3+} + O_2^-$ using the Applied Photophysics Pro-K software. Singular-value decomposition (SVD) and global fitting routines were used to derive basis spectra for the individual species and to compute rate constants.

2.7. Cryogenic UV–Vis spectroscopy

Ferric enzyme solutions were purged with N₂ at 4 °C and transferred to an N₂-filled glovebox for manipulation and preparation of the oxyferrous intermediate. All the buffers used in these procedures were thoroughly degassed using the aforementioned freeze–pump–thaw procedure. Anaerobic ferric mtCYP51 was reduced with a few crystals of sodium dithionite and incubated at 4 °C for 15 min. The UV–Vis absorption spectra from 350 to 700 nm were recorded in a sealed cuvette to confirm complete reduction as supported by the appropriate shift in the Soret band. After returning the sample to the glove box, excess sodium dithionite was removed using a Sephadex G-25 column previously equilibrated with anaerobic buffer. 0.25 mL Anaerobic ferrous enzyme was added to a polymethacrylate cuvette containing 0.75 mL of glycerol and thoroughly mixed. The final buffer composition was 50 mM Tris–HCl (pH 8.0) with 75% glycerol. Following a 1 h incubation at 4 °C, O₂ was bubbled into the cold glycerol/protein mixture for three minutes. The samples were subsequently frozen in liquid nitrogen vapors followed by flash freezing in the liquid. UV–Vis spectra were measured using an Olis Cary-14 spectrophotometer conversion instrument custom-modified to accommodate a Janis Research STVP-100 optical cryostat cooled to 80 K with liquid N₂.

2.8. Molecular dynamics simulations

Explicit-solvent MD simulations of mtCYP51, as well as the H259A and T260V mutants were performed as previously described [27,28] using the CHARMM22 force field [38] and the NAMD 2.9 code [39]. Before initiating simulations, a 5000 step conjugate gradient (CG) minimization was performed to eliminate unfavorable inter-atomic contacts. Water and ions were then equilibrated around the protein using NVT simulation for 500 ps with protein atoms fixed at their initial positions. The simulation was minimized for an additional 5000 CG steps and switched to the NPT ensemble to equilibrate protein with the solvent for a further 500 ps. Protein C_α atoms were restrained near their initial positions during this process using a 5.0 kcal·mol⁻¹·Å⁻² harmonic potential. Finally, all constraints were released and an NPT equilibration was performed for 30 ns to solvate and relax the system from its crystallographic conformation.

2.9. Oxygen diffusion calculations

The potentials of mean force (PMFs) for gas diffusion were obtained using the implicit ligand sampling (ILS) method [40,41]. This technique has previously produced physically plausible PMFs for monatomic and diatomic gas migration in systems including hydrogenases [42], oxygenases [43], and heme proteins such as myoglobin [40]. During ILS sampling, a small ligand (gas) molecule is placed at a series of regular grid points $\{r\}$ encapsulating the protein and the interaction energy $E(r)$ is computed, assuming that only van der Waals interactions contribute meaningfully through a Lennard–Jones interaction term. This process is repeated at each point for a total of M ligand conformations and sampled over N trajectory frames. The potential of mean force $A_{ILS}(r)$ at r can then be computed (Eq. (1)) at a temperature T where k is Boltzmann's constant. In practice, this affords a volumetric map from which isosurfaces may be produced at a given value of the PMF for a gas molecule.

$$A_{ILS}(r) = -kT \log \sum_{n=1}^N \sum_{m=1}^M \frac{\exp[-\Delta E_{m,n}(r)]}{NM} \quad (1)$$

Gas diffusion calculations were performed using data from the last 5 ns of NPT equilibration at 300 K for each system. Frames were sampled every 2.0 ps and ligand configurations sampled in 20 different orientations at each point on a 1.0 Å grid with 23 subsamplings at each grid point. CHARMM parameters were utilized to treat van der Waals interactions and ligand parameters for O₂ were assumed from prior, validated calculations [40]. PMF surfaces were constructed using the volmap utility as implemented in visual molecular dynamics [44].

3. Results and discussion

While the cognate substrate of mtCYP51 is unknown, it is nonetheless critical to have identified a model substrate of the enzyme for fruitful mechanistic studies. Two 14 α -methylated sterols, DHL and obtusifolol, have been identified as substrates for the enzyme. With support from spinach ferredoxin reductase and a mt ferredoxin to provide electrons, Bellamine and coworkers demonstrated that mtCYP51 removes the C14 methyl group from

both of these sterols, confirming that they both enter the enzyme active site and are subject to catalytic turnover [25]. While obtusifoliosol is demethylated at least five-fold more rapidly by mtCYP51 than DHL, this substance must be extracted from plant sources and is not readily available. In contrast, DHL can be prepared by simple and efficient hydrogenation of lanosterol (vide supra), which is commercially available at relatively low cost. We have therefore adopted DHL as the model substrate for the studies described herein.

Hallmarks of DHL and other ligands that do not coordinate to the heme iron of mtCYP51 are their common failure to induce a complete low- to high-spin transition of the heme iron that is measurable as a type-I spectroscopic response. In previous studies of mtCYP51, the fraction of enzymes converted to the high-spin state did not exceed 18% following titration with HPCD-encapsulated DHL [45]. X-Ray crystallography studies confirm that two additional ligands, estriol [46] and 4,4'-dihydroxybenzophenone [24], bind in the active site and their difference spectra support provocation of a more substantial spin transition than that observed for DHL. The observed spin transitions are nonetheless incomplete and likewise result in a mixture of the high- and low-spin species. While many compounds that provoke more substantial spin transitions are more convenient ligand binding studies, they must be confirmed as substrates before they can have potential as mechanistic probes.

3.1. UV-Vis spectral binding titrations

The Soret bands for all of the ligand-free, ferric CYP51 proteins occurred at 419 nm at 25 °C, consistent with low-spin, hexacoordinate aquaferric resting states of these enzymes. UV-Vis absorption difference spectra were used to monitor the low- to high-spin transition of the heme iron as the water ligand is displaced. Partial spin transitions were observed to depend on the concentration of DHL in all proteins with the exception of T260A. Titration of the equivalent concentrations of DHL-free HPCD does not result in a spin shift of any of the reported proteins. Despite the presence of 50 μ M DHL, the highest concentration used in these studies, T260A difference spectra provided no evidence for a change in the spin equilibrium. Analysis of the absolute spectra of ferric T260A revealed that the fraction of high-spin enzyme in the presence and absence of 50 μ M DHL were 27% and 29%, respectively. The apparent lack of spin shift or perturbation of the high-spin fraction by DHL supports that T260A is unlikely to be capable of accommodating DHL in the active site. On the other hand, mtCYP51, T260V, and H259A difference spectra displayed classic type-I difference spectra having troughs at 421, 422, and 422 nm and peaks at 388, 389, and 390 nm, respectively. The K_d determined for mtCYP51 was 0.20 μ M, somewhat lower than the 1 μ M value reported by Bellamine and coworkers [25]. Furthermore, with 50 μ M DHL present 15% of mtCYP51 was in the high-spin state, also similar to previous results [45]. The T260V and H259A mutants bind to DHL with somewhat lower affinity, having K_d values of 0.43 and 1.2 μ M, respectively. H259A behaved similar to mtCYP51, where at most 18% of H259A could be found in the high-spin state. This observation is not particularly surprising considering that the distance between H259 and the heme iron likely precludes its direct perturbation of the iron coordination sphere. The 36% high-spin T260V is expected since the critical T260 hydroxyl group capable of stabilizing the six-coordinate aquaferric enzyme is replaced by a methyl group. What was unexpected was that the spin shift depends on DHL. Thus, the origins of the change in spin equilibrium are more complex

than the loss of a hydrogen bond from T260. The equilibrium shift is more likely attributable to a ligand induced conformational shift of the valine residue and/or perturbation of the DHL binding mode.

Titration of CLO into all proteins results in a classic “type II” spectroscopic response, characterized by a red-shift of the Soret bands consistent with replacement of the water ligand with the CLOazole heterocycle. In difference spectra obtained by titration of CLO into mtCYP51, T260A, T260V, and H259A the Soret bands are shifted to 433, 429, 432, and 433 nm, respectively. The difference spectra of mtCYP51, T260V, and H259A share nearly identical troughs at 413–414 nm, consistent with depletion of the low-spin aquaferric enzyme by CLO. In contrast, this trough occurs at 392 nm in the T260A mutant demonstrating that CLO preferably depletes the high-spin enzyme population. K_d values for CLO were calculated by fitting the inverses of the absorbance changes and CLO concentrations. The K_d values of mtCYP51 (3.3 μ M), H259A (4.9 μ M), and T260A (4.1 μ M) are in approximate agreement with the 5 μ M value reported by Bellamine and coworkers [25]. This value was somewhat lower for the T260V (1.1 μ M) mutant.

3.2. Resonance Raman spectroscopy of ferric mtCYP51 and mutants

RR spectra were measured to investigate the changes in heme electronic structure resulting from HT mutations, DHL, andazole inhibitor binding. The high-frequency regions of the rR spectra from 406.7 nm laser excitation are illustrated in Fig. 2 and the frequencies of the major markers are compiled in Table 1. The high frequency region of the spectra contains bands that are sensitive to the oxidation (ν_4) and spin state (ν_3), those that are sensitive to the spin state and coordination environment (ν_2 and ν_{10}), and the stretching modes of the heme vinyl substituents ($\nu_{C=C}$). The oxidation state vibrations, ν_4 , occur at 1375 cm^{-1} in the spectra of mtCYP51 and T260A and at 1374 cm^{-1} in the spectra of T260V and H259A. These values are as expected for a P450 enzyme in the ferric oxidation state [33]. Binding of CLO whose imidazole group coordinates directly to the heme iron, shifts ν_4 at most 3 cm^{-1} . The spin-state sensitive bands measured for ligand-free and DHL-bound mtCYP51 are identical, each appearing at 1505 cm^{-1} (ν_3), 1586 cm^{-1} (ν_2), and 1642 cm^{-1} (ν_{10}). Collectively, these markers are characteristic of the low-spin, hexacoordinate aquaferric enzyme. The high-spin component of the mtCYP51-DHL complex observed in the UV-Vis spectra was not visible in the rR, possibly due to the smaller Raman cross sections of the high-spin markers. CLO binding to mtCYP51 consistently red shifts the ν_3 , ν_2 , and ν_{10} bands. The largest of these is the 8 cm^{-1} shift of ν_{10} , possibly attributable to ligand charge transfer. Charge transfer from the CLO ligand would weaken the C_α - C_{meso} π bonds of the heme, thereby decreasing the frequency of ν_{10} . In mtCYP51, DHL has a negligible effect on $\nu_{C=C}$, while CLO shifts these to 1619 cm^{-1} . Regardless of their initial positions in the spectra, CLO likewise shifts the $\nu_{C=C}$ band to the same position in the spectra of the mutants.

The high frequency rR spectra of the mutants confirm the results of UV-Vis absorption studies that lesions of the HT dyad alter the spin equilibrium of the ligand-free enzyme and change the sensitivity of the spin equilibrium to ligand binding. T260A exemplifies the former scenario in which the enzyme is partially in the high-spin configuration in the ligand

free form. Furthermore, the spectrum remains essentially unchanged following the addition of DHL. This observation underscores the conclusions of the difference spectra that T260A does not bind to DHL. The predominant ν_3 band in the T260A spectra occurs at 1504–1505 cm^{-1} , consistent with the low-spin form of the enzyme; however these bands have a shoulder centered at 1489 and 1490 cm^{-1} revealing the high-spin subpopulation of the enzyme observed in the UV–Vis spectra. By contrast, binding of CLO abolishes the ν_3 shoulder from the high-spin component and shifts the remainder of the spectrum to mirror that of the low-spin mtCYP51–CLO complex. While the rR spectrum of T260V in its unbound form reflects that of ligand-free mtCYP51, the T260V spin state markers and $\nu_{\text{C}=\text{C}}$ bands undergo substantial shifts on binding to DHL. The ν_3 , ν_2 , and ν_{10} bands in the DHL-free enzyme occur at 1504, 1587, and 1641 cm^{-1} , respectively and are consistent with the low-spin enzyme. In the presence of DHL however, there are marked shifts of the ν_3 and ν_2 bands to 1487 and 1570 cm^{-1} , respectively, both characteristic of the high-spin form. Thus, the T260V mutant behaves somewhat similarly to P450cam [33], where substrate binding largely displaces the axial-coordinated water molecule resulting in the pentacoordinate, high-spin enzyme. Furthermore, the $\nu_{\text{C}=\text{C}}$ band of this mutant is the most sensitive to DHL, which induces a 7 cm^{-1} shift in accord with change in the environment surrounding the heme vinyls. DHL binding to H259A similarly induces a partial low- to high-spin transition, though it is less pronounced than that observed with T260V. The CLO-bound forms of both T260V and H259A reflect those of the mtCYP51–CLO complex and are similarly in the low-spin state.

The low frequency regions of the rR spectra are illustrated in Fig. 3 and the frequencies of the major markers are compiled in Table 1. The low frequency region contains the heme skeletal stretching modes as well as bending vibrations of the peripheral vinyl and propionate moieties. As is typical in the rR spectra of P450 enzymes, the heme stretching modes ν_7 and ν_8 , centered at 675 and 343 cm^{-1} are resistant to mutations and ligand binding. In mtCYP51, the propionates bending mode ($\delta_{\text{propionates}}$) appears at 380 cm^{-1} and the peripheral vinyl bending mode (δ_{vinyl}) appears at 409 cm^{-1} . These assignments are made based on the complete vibrational assignments of the heme in myoglobin and cytochrome c [47,48]. Changes in the chemical environments surrounding these functional groups are reflected in shifts of the corresponding frequencies in the rR spectrum. The $\delta_{\text{propionates}}$ frequency positions provide insight into the hydrogen bonding and/or ionic interactions surrounding the heme propionates that anchor the cofactor into the active site. The δ_{vinyl} frequencies are sensitive to the coplanarity of the vinyl functional group with the heme. Out-of-plane vinyls generally have higher δ_{vinyl} than those that are coplanar with the heme macrocycle. The δ_{vinyl} frequency decrease is due to the enhanced with-drawing of electron density from the vinyl when it is in the heme plane, as opposed to an increase in this frequency when the overlap between the vinyl and π -electron systems are attenuated when the vinyls are nearly perpendicular to this plane [49,50]. In mtCYP51, DHL only induces a 1 cm^{-1} shift of δ_{vinyl} and a 3 cm^{-1} shift of $\delta_{\text{propionates}}$, supporting that this ligand does not induce marked conformational or environmental changes of the heme peripheral substituents. Mirroring the $\nu_{\text{C}=\text{C}}$ band shifts observed in the high frequency region, CLO binding shifts the δ_{vinyl} vibrations by 11 cm^{-1} to 420 cm^{-1} . This observation is in accord with the previous results of Matsuura and coworkers who observed a 13 cm^{-1} shift of the

δ_{vinyl} vibration with ketoconazole, 4-phenylimidazole, and fluconazole [51]. Matsuura and coworkers did not observe a corresponding shift of the $\delta_{\text{propionates}}$ and thus concluded that the azoles did not interfere with the heme propionates. For comparison, we measured the rR spectra of mtCYP51 with 300 μM fluconazole and ketoconazole. The measured values of $\delta_{\text{propionates}}$ and δ_{vinyls} at 385 cm^{-1} and 426 cm^{-1} respectively were identical to the previously reported measurements [51]. Shifts observed in the δ_{vinyl} vibrations of mtCYP51 spectra uniformly support that the azole ligands shift the vinyls out of the heme plane. There are corresponding shifts of the $\nu_{\text{C}=\text{C}}$ to higher frequencies in the high-frequency region of these spectra; however there is evidence opposing that there is a correlation between $\nu_{\text{C}=\text{C}}$ and the orientation of the vinyl group relative to the heme plane [52] and so these are excluded from further analyses of vinyl conformations. Moreover, these spectra similarly lacked evidence of perturbations to the heme propionate environments. However, in the spectra of CLO-mtCYP51 the dominant $\delta_{\text{propionates}}$ band at 379 cm^{-1} has an obvious shoulder at 367 cm^{-1} . Thus, the observation of a second, overlapping band confirms that CLO imparts conformational heterogeneity to the heme propionates. Furthermore, the magnitude (12 cm^{-1}) of this partial shift supports that the number and/or strength of the hydrogen bonds differ among the accessible configurations of the mtCYP51–CLO complexes. A 10 cm^{-1} shift is typical for the cleavage of a propionate hydrogen bond as demonstrated for thromboxane synthase and myoglobin [53,54]. In summary, DHL does not induce perceptible changes in the conformations and/or environments of the heme peripheral substituents, both fluconazole and ketoconazole only perturb the heme vinyls, and CLO uniquely perturbs both the vinyls and the propionates.

rR studies of CLO and DHL binding to the mutants revealed that while many of the effects on the heme periphery were similar to the corresponding mtCYP51 complexes, some notable differences were observed. The effects of CLO on both the δ_{vinyls} and $\delta_{\text{propionates}}$ are ubiquitous. CLO red shifts the δ_{vinyl} vibrations by 17 cm^{-1} , 10 cm^{-1} , and 12 cm^{-1} in the T260A, T260V, and H259A mutants, respectively. The dominant $\delta_{\text{propionates}}$ bands appear at 377, 379, and 379 cm^{-1} in the T260A, T260V, and H259A mutants, respectively, in accord with this ligand uniformly inducing out-of-plane conformations of the heme vinyl groups. Like mtCYP51, these have shoulders at 364, 369, and 367 cm^{-1} , so the apparent conformational heterogeneity induced by CLO is conserved among all of the CLO–enzyme complexes. In response to DHL binding, changes to the low frequency are subtle though nonetheless significant. Compared to ligand-free mtCYP51, only the $\delta_{\text{propionates}}$ of T260A shift from 4 cm^{-1} to 384 cm^{-1} . In contrast, the vinyl and propionate vibrational markers of T260V are remarkably sensitive to DHL binding. On binding DHL, the T260V $\delta_{\text{propionates}}$ intensity at 381 cm^{-1} decreases and shifts to 378 cm^{-1} while a new band appears at 364 cm^{-1} . Apparently, the $\delta_{\text{propionates}}$ of T260V behave similarly to when CLO is present by shifting to a frequency telling of substantial changes in the hydrogen bonding environments surrounding the propionates. DHL binding to T260V likewise shifts the δ_{vinyl} marker from a relatively sharp band at 410 cm^{-1} to a broad δ_{vinyl} centered at 424 cm^{-1} consistent with DHL inducing conformational heterogeneity into the vinyls with concurrent loss of coplanarity between the vinyls and the heme plane. Taken together with spectra of the high frequency region, both the spin state and heme conformation are sensitive to DHL in the active site. Finally, the low frequency rR spectrum of the H259A mutant reflects that of the

wild type; however, there are minor 2 cm^{-1} and 3 cm^{-1} shifts of the $\delta_{\text{propionates}}$ and δ_{vinyl} modes supporting that this mutation causes only minor conformational or environmental changes of the heme peripheral groups. It appears then that the mutants each respond similarly to mtCYP51 on binding CLO, however in contrast HT mutations result in a continuum of sensitivity of the heme peripheral environment to DHL.

3.3. MD simulations

MD simulations were performed in explicit water to explore the conformational landscapes of the amino acid side chains bounding the active site, DHL, and the heme cofactor to understand the structural underpinnings for the rR observations. On the timescale of the simulations, these proteins maintain their overall fold and no major conformational changes were observed. There are however visible differences in the orientation of DHL and the environments of the heme propionates. Illustrated in Fig. 4A, the final trajectory point from the 30 ns simulations were superimposed to illustrate the relative position of DHL and the chemical environments of the heme propionates following MD equilibration. All simulations illustrate that the 17β -aliphatic chain of DHL is oriented above the propionates while the steroid skeleton lies above the porphyrin. The binding modes of DHL in mtCYP51 and H259A are quite similar, differing primarily in the conformation of the 17β -chain. In T260V however, DHL is shifted away from the propionates along the extended axis of the molecule and upward toward the roof of the active site. Our expectation was that the valine side chain would occupy a similar volume and spatial orientation as that of threonine. To our surprise, the MD simulations showed that the probable orientation of the valine methyl is oriented away from the iron and toward the heme periphery. This orientation places the additional methyl group adjacent to the heme 2-vinyl, resulting in a migration of the vinyl group below the heme plane. This prediction is validated in the rR spectra of this mutant, in which DHL binding induces a 14 cm^{-1} shift of the δ_{vinyl} frequencies, consistent with the departure of at least one of the heme vinyls from the heme plane. Illustrated in Fig. 4B, simulations also predict that HT mutations result in environmental changes around one or both of the heme propionates. In the T260V-DHL complex, hydrogen bonds between the 7-propionate, Q72 and H392 are disrupted that are otherwise present in mtCYP51 or H259A. Rearrangement of the amino acids observed in the MD simulations are corroborated by the magnitude of shifts observed in rR spectra. In the T260V mutant, simulations predict the disruption of two hydrogen bonds, consistent with the large shift observed in the propionate vibrations. In the H259A-DHL complex, only H101 moves away from the 6-propionate. The minor reorientation of residues observed in the H259A simulation are consistent with the correspondingly small vibrational shifts observed in the rR spectra.

3.4. Oxyferrous formation and decay kinetics

The oxyferrous species is the first important branch point in P450 catalysis from which the catalytic cycle can proceed to productive monooxygenation of substrate or uncouple to afford the ferric enzyme and superoxide anion. The oxyferrous has been characterized in a few cytochrome P450 and only in those bearing the conserved acid-alcohol pair. To establish the CYP51 mechanism, it is critical that we first understand the impact of the active site on the stability of the oxyferrous complex. To this end, we have measured the

rates of oxygen binding (k_1) and auto-oxidation (k_2) of oxyferrous mtCYP51 and the HT mutants. These rate constants are compiled in Table 1.

For comparison to existing literature and to validate the approach in our hands, these rates were also measured for CYP101 in the presence of 1 mM camphor. Spectra collected after mixing ferrous CYP101 with oxygen-saturated buffer and the basis spectra resulting from SVD analysis are illustrated in Fig. 5A and B. Oxyferrous CYP101 displays a Soret band at 421 nm and a single peak at 555 nm in the vicinity of the α and β absorption bands. The decay of these spectroscopic features correspond to a shift of the Soret band to 391 nm, consistent with the formation of the high-spin, camphor-CYP101 complex and the superoxide anion. In experiments with CYP101, the ferrous enzyme was rapidly depleted within the dead time of the instrument precluding the calculation of a reliable rate constant for O_2 binding. The rate constant measured for auto-oxidation of the oxyferrous enzyme was 0.0067 s^{-1} at $25\text{ }^\circ\text{C}$. This value is nearly identical to the 0.0074 s^{-1} value measured by Eisenstein and coworkers in their stopped-flow kinetic analysis at $26\text{ }^\circ\text{C}$. Moreover, these CYP101 values are consistent with the literature values of 0.004 s^{-1} measured at $20\text{ }^\circ\text{C}$ [55] and 0.0043 s^{-1} measured by Peterson and coworkers at $23\text{ }^\circ\text{C}$ [56].

In the mtCYP51 stopped flow experiments, the spectra of ligand-free ferrous enzymes were collected over 0.5 s (400 scans/s) after mixing the anaerobic, ferrous enzyme with O_2 -saturated buffer. Unlike the ferric enzymes that are represented by a mixture of spin states, UV-Vis spectra support the presence of a single ferrous spin state. Both mtCYP51 and mutants have a single Soret band at 424–425 nm and α - and β -bands at 528 and 558 nm, respectively regardless of whether DHL is present. Spectroscopic evidence for the single ferrous spin species justifies the use of the $Fe^{2+} \xrightarrow{k_1} Fe^{3+}O_2^- \xrightarrow{k_2} Fe^{3+} + O_2^-$ kinetic model. Spectra for wild-type mtCYP51 in the presence and absence of DHL are illustrated in Fig. 5C–F. After mixing, the Soret band shifts from 424 nm (at 2.5 ms) to 418 nm (0.5 s), consistent with conversion of the ferrous to the low-spin ferric enzyme. In addition, SVD analysis reveals a third basis vector with a Soret band at 422 nm whose accumulation and decay coincide with the formation and dissipation of the oxyferrous enzyme and the appearance of the ferric enzyme. This basis vector represents the transient accumulation of the oxyferrous intermediate. The pseudo-first order rate constant for the formation of oxyferrous mtCYP51 is $63\text{ }\mu\text{M}^{-1}\cdot\text{s}^{-1}$, while the presence of DHL attenuates k_1 to $15\text{ }\mu\text{M}^{-1}\cdot\text{s}^{-1}$. The results of previous MD simulations Published by our group [28] support that DHL largely occludes the putative substrate entrance channel [57]. We speculate that the rate constant for DHL-free mtCYP51 corresponds to the fully “open” form of the enzyme. This assertion is founded in the crystal structures of ligand-free ferric mtCYP51, where the BC-loop that bounds the access channel is disordered [46,58]. Assuming that this channel is also the predominant pathway for O_2 diffusion into the active site, it is not surprising that the presence of DHL reduces the O_2 binding rate. The presence of DHL has little impact on the rate of oxyferrous mtCYP51 auto-oxidation. This behavior is quite different from that exhibited by other P450 enzymes. For example, the removal of camphor from CYP101 increased the auto-oxidation rate constant ~3.5-fold at $26\text{ }^\circ\text{C}$ [55]. Likewise, Denisov and coworkers reported that the auto-oxidation of nanodisc-incorporated CYP3A4 increased 60-

fold following the removal of testosterone and 150-fold following the removal of bromoergocryptine [59].

HT mutations do change the O₂-binding and oxyferrous oxidation kinetics in a ligand dependent manner. In accord with the previous evidence that DHL does not bind to T260A, the rate constants for O₂ binding are $\sim 31 \mu\text{M}^{-1}\cdot\text{s}^{-1}$, regardless of the presence DHL. This rate is only half that determined for mtCYP51, therefore this mutation must alter the primary O₂ diffusion pathway. Since DHL is expected to occlude the primary O₂ diffusion pathway in these enzymes, increases in O₂ binding rates for both T260V-DHL and H259A-DHL seemed counterintuitive. The rate of O₂ binding to the T260V is reduced approximately four-fold relative to that of the mtCYP51. Interestingly, the rate for T260V-DHL O₂ binding is twice that determined for mtCYP51-DHL. The O₂ binding rate constant to H259A is attenuated by half relative to mtCYP51; however, the rate in the presence of DHL is among the largest measured in these studies and comparable to DHL-free mtCYP51. We speculate that these mutations stymie a DHL-induced conformational shift that would otherwise partially occlude the primary O₂ diffusion pathway. Nevertheless, we use the implicit ligand sampling method, described below, to glean insight into the structural bases for these seemingly paradoxical rates. Unlike those for O₂ binding, the rates of oxyferrous auto-oxidation are mostly $\sim 2\text{--}3 \text{ s}^{-1}$. The single exception in these studies is the T260V-DHL complex. In this case, the observed rate is nearly three-fold greater than that measured for the mtCYP51-DHL complex. It is tempting to attribute the relative instability of this oxyferrous species to the loss of the stabilizing effect imparted by a T260 hydrogen bond and MD simulations support that this effect may be at least partially due perturbation of the heme geometry. Such changes in the electronic structure of the heme iron could weaken the Fe–O₂ bond of the incipient oxyferrous species, explaining the elevated auto-oxidation rate observed in T260V-DHL. Since HT mutations undoubtedly affect the architecture of the active site to afford changes in the rates of O₂ binding and auto-oxidation, it is not prudent to interpret these results based on local property changes induced by replacing amino acids in the mtCYP51 sequence alone. Taken together with the rR measurements, these results support that there are widespread changes in the active architecture induced by HT mutations.

3.5. Cryogenic UV–Vis absorption spectroscopy

To verify that the SVD-derived absorption spectra correspond to authentic intermediates, we measured the UV–Vis spectra of the ferric, ferrous, and the oxyferrous species at 80 K in aqueous-glycerol matrices. While spectra of the ferric and ferrous enzymes can be easily measured at ambient temperature, the half-life of the oxyferrous species is at most 0.38 s at 25 °C (H259A; Table 2), which precludes using the standard measurement techniques. The oxyferrous intermediates were prepared by bubbling O₂ into buffer containing 75% glycerol at 4 °C, followed by freezing in liquid N₂. The large glycerol content affords an optically transparent glass that when frozen is ideal for optical spectroscopy at low temperatures [60,61]. If the cold oxyferrous samples were allowed to anneal to room temperature, they rapidly auto-oxidized to the ferric oxidation state. Ferrous samples could be annealed to room temperature without appreciable oxidation to the ferric state. The Soret band positions for each species with and without DHL are compiled in Table 3. The complete cryogenic

spectra of mtCYP51 and mtCYP51-DHL are illustrated in Fig. 6, while those of the mutants are available in Figure S5 of the Supplementary Data. In general, the spectra of the ferric, ferrous, and oxyferrous species in frozen aqueous glycerol matrices are very similar to those assigned to the basis vectors resulting from the SVD calculations. In close agreement with the kinetic analysis, oxyferrous, ferrous, and ferric mtCYP51 have Soret bands at 424, 422, and 418 nm, respectively while those for the mtCYP51-DHL complex are at 420, 422, and 418 nm. The minor differences between the cryogenic spectra and SVD basis vectors may be attributed to the high glycerol content of the buffer and the reduction in temperature broadening of the Soret band at low temperature. The behavior of the ferric state of the T260V mutant in the presence of DHL deviates from this behavior. At 80 K, the Soret band of the T260V mutant with DHL is split, having maxima at 395 and 415 nm characteristic of a mixture of the high- and low-spin states of the heme iron. Since the high-spin component is not visible at ambient temperature, this observation supports that in the presence of DHL, the low-spin configuration is an electronic excited state of the heme core. The percentage of T260V in the high-spin state at 80 K is 55%. The increase in high-spin content as the temperature is lowered indicates that the high-spin state is actually the ground electronic state of the T260V-DHL complex. Hence, the energetic ordering of the high- and low-spin states is inverted in the T260V mutant and this observation may be attributable to the predicted distortion of the heme macrocycle in this mutant. The T260V mutant could therefore prove useful as a model system in future studies of P450 spin equilibria.

3.6. Oxygen diffusion calculations

To reconcile the observations made in the stopped-flow kinetic experiments measuring O₂ binding, we computed three-dimensional PMFs for O₂ diffusion using the ILS approach and the final 5 ns of the MD simulations. This method provides a comprehensive overview of how mutation-induced side chain dynamics affect the O₂ diffusion pathways and their energetic barriers. Owing to the computational expense of the ILS sampling method for these systems and our primary interest in pathways available for O₂ diffusion into the DHL complexes, we limited our simulations to the mtCYP51-, T260V-, and H259A-DHL complexes. Illustrated in Fig. 7, the ILS simulations indicate that there is a common, predominant path for O₂ transport into each of the CYP51 proteins (Fig. 7, A, C, and F). This common channel extends from the protein surface adjacent to A73 in the loop on the N-terminal side of the B'-helix, above the heme propionates, terminating at the heme iron under the C and D rings of DHL. While continuous pathways are evident in simulations of both the WT and H259A mutants, only three disconnected regions consistent with such a pathway are visible at a contour value of 2.5 kcal mol⁻¹ in simulations of the T260V mutant. A second channel begins at the protein surface and terminates prematurely between the H259 and T260 residues of mtCYP51 and T260V mutant (Fig. 7B and F). In the case of the T260V mutant, it is possible that the length of the simulation was not sufficient to capture the opening of a second channel to support the observed O₂ binding rate. Conversely, in the H259A mutant, this second channel extends deep into the active site and terminates near the heme iron flanked by the A/B rings of the substrate and T260 (Fig. 7D). The presence of this second O₂ migration pathway with a comparable free energy barrier for diffusion as the common pathway explains the accelerated rate observed in the H259A-DHL complex. The opening of this channel is afforded by the loss of an ion pair between H259 and E173 by

replacing the former residue with alanine. Moreover, in H259A this second channel is populated by a hydrogen-bonded network of water molecules that extends from bulk solvent at the protein surface to the heme (Fig. 7E), reinforcing that molecules of similar size to O₂ can exploit this channel to gain entry in to the enzyme active site.

4. Summary and conclusions

We have characterized mtCYP51 and three variants bearing mutations in the HT dyad and demonstrate the utility of concomitant MD simulations to interpret disparate spectroscopic observations in the context of active site structure. Evidenced by similar binding affinities for DHL and CLO, the mutations are well tolerated. RR demonstrates the changes of the heme's electronic structure, conformations of the peripheral groups, propionate environment, and DHL binding orientation. The greatest perturbations are observed in the T260V mutant. T260V active site changes are evident in the relevant rR vibrational signatures and UV–Vis spectra at 80 K. The conclusions of these spectroscopic studies are supported by the results of molecular dynamics simulations. Despite the apparent conservation of volume when mutating threonine to valine, in this case the result is a comprehensive change in the heme environment.

This study also describes the kinetic studies of O₂ binding to the ferrous enzymes as well as auto-oxidation of the corresponding oxyferrous intermediates. The decrease in the O₂ binding rate in mtCYP51 in the presence of DHL is not surprising, since the ligand is expected to occlude a prominent diffusion pathway. Paradoxically, T260V–DHL and H259A–DHL demonstrate two- and four-fold larger rate constants for O₂ binding than mtCYP51–DHL. The ILS of the respective MD trajectories revealed the probable structural bases for these observations. These clearly illustrated alternative O₂ diffusion pathways into the T260V and H259A active sites. This application of the ILS approach demonstrates its value in supporting the interpretation of kinetic studies of diatomic molecule diffusion into proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CCD	charge-coupled device
CLO	clotrimazole
CG	conjugate gradient

CYP51	sterol 14 α -demethylase
CYP101	cytochrome P450cam
DHL	24,25-dihydrolanosterol
HPCD	2-hydroxypropyl- β -D-cyclodextrin
FLU	fluconazole
ILS	implicit ligand sampling
KET	ketoconazole
LB	Luria–Bertani
MD	molecular dynamics
mt	<i>Mycobacterium tuberculosis</i>
NAMD	nanoscale molecular dynamics
NPT	isobaric–isothermal ensemble
PMF	potential of mean force
NVT	canonical ensemble
rR	resonance Raman
SVD	singular-value decomposition

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jinorgbio.2014.03.017>.

Fig. 1. Superimposition of the mtCYP51 (orange, lighter color) and CYP101 (blue, darker color) active sites illustrating the positions of the CYP51 H259–T260 dyad and CYP101 “acid–alcohol” pair (D251–T252) relative to the hemes. PDB accession numbers 2VKU and 1DZ8 for mtCYP51 and CYP101 were used, respectively.

Fig. 2.

High frequency regions of the resonance Raman spectra of mtCYP51 and mutants. Spectra were collected using 406.7 nm laser excitation. Spectra of mtCYP51 (A), T260A (B), T260V (C), and H259A (D) each utilized a 10 μ M protein concentration. Spectra of ligand-free proteins are depicted in blue, 50 μ M DHL in green, 25 μ M clotrimazole in red, 300 μ M fluconazole in purple, and 300 μ M ketoconazole in black.

Fig. 3.

Low frequency regions of the resonance Raman spectra of mtCYP51 and mutants. Spectra were collected using 406.7 nm laser excitation. Spectra of mtCYP51 (A), T260A (B), T260V (C), and H259A (D) each utilized a 10 μ M protein concentration. Spectra of ligand-free proteins are depicted in blue, 50 μ M DHL in green, 25 μ M clotrimazole in red, 300 μ M fluconazole in purple, and 300 μ M ketoconazole in black.

Fig. 4. Superimposition of the final trajectory frame of the MD simulations. (A) The binding modes of DHL in mtCYP51 (green), T260V (yellow), and H259A (purple). (B) Residues within hydrogen-bonding distance (1.5 Å) of the heme propionates. MtCYP51, T260V, and H259A are represented in green, yellow, and purple respectively.

Fig. 5.

Stopped-flow reactions of CYP101 with 1 mM camphor (A) mtCYP51 (C) and mtCYP51 with 50 μM DHL (E). The corresponding spectra of oxyferrous and ferric CYP101 (B), ferric, ferrous, and, oxyferrous mtCYP51 (D) and ferric, ferrous, and, oxyferrous mtCYP51 with 50 μM DHL (F) derived from SVD analysis. An enlarged view of the Soret band maxima are illustrated in the inset. The reaction progress curves illustrating the concentrations of each species are inset into panels B, D, and F. The protein concentrations were 10 μM. Four-hundred spectral traces were collected per second.

Fig. 6.

UV-Vis spectra of the ferric, ferrous, and oxyferrous states of 10 μ M mtCYP51 in aqueous glycerol matrices at 80 K. Spectra were collected in 50 mM Tris-HCl (pH 8.0) with 75% (v/v) glycerol in the absence (panel A) and presence of 50 μ M DHL (panel B).

Fig. 7.

Implicit ligand sampling PMF maps for O_2 migration in mtCYP51, H259A, and T260V. The common O_2 migration pathways for mtCYP51, T260V and H259A are illustrated in panels A, C, and F, respectively. The prematurely terminating channel in mtCYP51 is illustrated in panel B. The second O_2 migration channel in H259A and the occupant water molecules are illustrated in panels D and E, respectively. Isosurface maps are illustrated at $2.5 \text{ kcal mol}^{-1}$.

Scheme 1.

Table 1

Frequencies (cm^{-1}) of the relevant vibrational markers in the resonance Raman spectra obtained using 406.7 nm laser excitation of 10 μM mtCYP51, HT mutants, and their complexes with ligands.

	T260A				T260V				H259A			
	mtCYP51		+KET		+DHL		+CLO		+DHL		+CLO	
	Unbound	+DHL	+CLO	+FLU	Unbound	+DHL	Unbound	+CLO	Unbound	+DHL	Unbound	+CLO
ν_8	343	343	343	345	343	343	345	345	343	342	344	344
δ_{prop}	380	383	379	385	380	384	377	379	381	378	384	379
			367				364	369		364		367
δ_{vinyls}	409	410	420	426	407	409	424	420	410	424	409	412
ν_7	675	674	675	676	675	675	674	674	676	674	675	674
ν_{15}	753	751	752	752	752	753	750	752	753	752	753	751
ν_4	1375	1375	1373	1373	1375	1375	1373	1373	1374	1375	1374	1372
ν_3	1505	1505	1501	1502	1505	1504	1501	1501	1504	1504	1504	1500
					1489	1490			1487		1486	
ν_2	1586	1586	1584	1585	1586	1587	1584	1584	1587	1584	1585	1584
									1570			
$\nu_{\text{C}=\text{C}}$	1629	1628	1619	1622	1624	1624	1619	1619	1632	1625	1627	1620
					1631	1631						
ν_{10}	1642	1642	1634	1637	1640	1642	1634	1635	1641	1643	1641	1634

Table 2

mtCYP51 and mutant ferrous O₂-binding (k_1 , $\mu\text{M}^{-1}\cdot\text{s}^{-1}$) and oxyferrous auto-oxidation (k_2 , s^{-1}) rate constants at 25 °C.^a

	<u>-DHL</u>		<u>+DHL</u>	
	k_1	k_2	k_1	k_2
mtCYP51	63 ± 4	2.1 ± 0.1	15 ± 0.9	2.6 ± 0.1
T260A	31 ± 5	3.4 ± 0.5	32 ± 3	2.9 ± 0.1
T260V	16 ± 3	2.1 ± 0.1	32 ± 6	7.3 ± 1.3
H259A	36 ± 4	2.0 ± 0.1	68 ± 1.3	1.8 ± 0.1
CYP101 ^b	$6.7 \times 10^{-3} \pm 7 \times 10^{-5}$			

^a Mean (n = 3) ± standard deviation.

^b In the presence of 1 mM camphor.

Table 3

Soret band positions (nm) in the UV–Vis spectra of ferric, ferrous, and oxyferrous mtCYP51 and mutants derived from SVD analysis of stopped-flow scans and from cryogenic spectroscopy at 80 K.

mtCYP51	<u>Singular-value decomposition</u>		<u>Aqueous glycerol glass at 80 K</u>	
	–DHL	+DHL	–DHL	+DHL
WT	416,425,421	418,421,420	418, 424,422	418, 422, 420
T260A	416,424,423	418,425,422	416,424,423	416,425,424
T260V	416,424,423	420,425,422	418,424,423	394 & 415, 424, 422
H259A	420,425,422	421,421,425	418,424,422	418,425,424