The Structure of Mycobacterium tuberculosis CYP125
MOLECULAR BASIS FOR CHOLESTEROL BINDING IN A P450 NEEDED FOR HOST INFECTION*

We report characterization and the crystal structure of the Mycobacterium tuberculosis cytochrome P450 CYP125, a P450 implicated in metabolism of host cholesterol and essential for establishing infection in mice. CYP125 is purified in a high spin form and undergoes both type I and II spectral shifts with variousazole drugs. The 1.4-Å structure of ligand-free CYP125 reveals a “letterbox” active site cavity of dimensions appropriate for entry of a polycyclic sterol. A mixture of hexa-coordinate and penta-coordinate states could be discerned, with water binding as the 6th residue. Structures in complex with androstenedione and the anti-tuberculosis drug BETO revealed structural and catalytic properties of a potential drug target enzyme, and the likely mode by which the host-derived substrate is bound and hydroxylated.

The global threat to human health posed by the bacterium Mycobacterium tuberculosis (Mtb) was recognized by the World Health Organization some years ago (World Health Organization fact sheet on “Tuberculosis” located online at: www.who.int/mediacentre/factsheets/fs104/en), and it is estimated that one-third of the world’s population is infected with the Mtb bacillus. Synergy with the HIV virus, failures in drug administration to patients, and the consequences of the development of drug and multidrug-resistant strains of Mtb have made the situation ever more perilous and it is widely acknowledged that novel intervention strategies are needed (1).

The determination of genome sequences of Mtb strains led to revelations relating to the protein repertoire of the pathogen, and highlighted the large number of enzymes involved in lipid metabolism (2, 3). Mtb has an extraordinary array of complex lipids, including unusual long chain, extensively substituted lipids (mycolipids) that form a waxy coat around the bacterium and are likely important in preventing antibiotic entry (4). Another interesting observation relating to lipid metabolizing enzymes is the large number of Mtb cytochrome P450 (P450 or CYP) enzymes. P450s are heme-containing monooxygenases, well known for their roles in metabolism of fatty acids, steroids, and other lipophilic molecules (5). This suggests there may be critical roles for a number of these enzymes in Mtb lipid metabolism (6). Consistent with this theory, gene disruption and gene deletion studies have, to date, shown that Mtb CYP121 and CYP128 are essential genes for cell growth and viability (7, 8). These P450s have recently been proposed to have roles in C–C bond formation in a cyclic dipeptide and in hydroxylation of respiratory menaquinone, respectively (9, 10). Although physiological roles for many Mtb P450s remain unclear, Mtb CYP51B1 has been structurally and biophysically characterized, and catalyzes demethylation of various sterols (11, 12). This activity is consistent with that of eukaryotic CYP51 enzymes, suggesting that CYP51B1 has roles in host sterol metabolism. Importantly, it was demonstrated that various azole drugs (that inhibit fungal CYP51 by coordinating the heme iron) are also potent inhibitors of mycobacterial growth, thus suggesting that one or more Mtb P450s may be azole targets (13–15). Econazole and other azoles bind tightly to various Mtb P450s, including CYP121, CYP125B1, and CYP130 (10, 11, 13, 16, 17). Econazole is effective in clearing Mtb infection in a mouse model, and recent studies on Mtb CYP130 (a P450 whose gene is deleted in the vaccine strain Mycobacterium bovis BCG) revealed the binding mode of the drug to this P450 (16, 18).
Recently, a gene cluster in *Rhodococcus* sp. strain RHA1 was identified as being involved in catabolism of cholesterol (19). Several of these genes are conserved in Mtb, including the P450s CYP125 and CYP142 (20), suggesting that these have roles in cholesterol (or possibly other sterol) metabolism. Early studies of the protein interactions of the Mtb CYP125 with nitric oxide indicated that its ferrous-nitric oxide complex was relatively labile, and thus that CYP125 may be relatively resistant to macrophage-generated nitric oxide (21). Transcriptomic studies showed that Mtb H37Rv CYP125 is induced in macrophages, and it is reported to be essential for infection of mice; one of only 26 genes present in both categories (22). Furthermore, cholesterol, along with the phagosomal tryptophan-aspartate-containing coat protein, is crucial for Mtb entry into the macrophage and for establishment of intracellular infection by Mtb (23). In other work, genetic inactivation of the Mtb cholesterol oxidase (ChoD) resulted in attenuation of the choD mutant strain, implicating ChoD in Mtb pathogenesis (24). Also, recent studies implicated the actinobacterial *mcu* gene locus (conserved in Mtb) with cholesterol/steroid uptake (25). Finally, it was shown that Mtb uses cholesterol as a source of carbon and energy for growth, suggesting that exploitation of host cholesterol may underlie persistence and survival in humans (26).

To investigate properties of the CYP125 P450 from the putative Mtb “cholesterol cluster,” we have purified Mtb CYP125 heterologously expressed in *Escherichia coli* and explored its thermodynamic and spectroscopic features, including its ligand-binding properties. We have determined the CYP125 crystal structure in a ligand-free state and in complex with econazole and androstenedione. Generation of a molecular model of the cholesterol complex indicated that cholesterol C25 and the terminal methyl (C26/27) carbons are exposed to the heme iron. Turnover studies demonstrated conclusively that CYP125 is a cholesterol 27-hydroxylase. Our data suggest a key role for CYP125 in Mtb cholesterol metabolism as a C27 hydroxylase, and thus its importance in infectivity and in persistence of Mtb in the human host.

**EXPERIMENTAL PROCEDURES**  
CYP125 Cloning, Expression, and Purification—CYP125 was cloned by PCR from a Mtb H37Rv cosmid library (from Institut Pasteur, Paris). The BAC clone containing CYP125 (Rv3545c) was prepared by standard protocols, and used as template DNA for the PCR using *Pfu* Turbo DNA Polymerase (Stratagene) and the oligonucleotide primers designed from the Mtb genomic sequence: upstream 5’-GGACAGGATATGTCGGAGATCCACGTCGTTACGTCA-3’ and downstream 5’-CAGTGGGATAGTC- TCCATTAGTGAGCAAC-3’. The bold letters in the upstream primer indicates an engineered NdeI restriction cloning site, and downstream 5’-GGACAGGATATGTCGGAGATCCACGTCGTTACGTCA-3’ and downstream 5’-CATGACGCAAC-3’. The underlined letters in the downstream primer indicate a BglII restriction cloning site. Amplification conditions were 95 °C for 2 min, 30 cycles of 95 °C for 50 s, 63 °C for 30 s, and 72 °C for 2 min, followed by a final polymerization step of 72 °C for 8 min. CYP125 was cloned into pET15b (Merck) using the NdeI and BamHI restriction sites and using the compatible cohesive ends between BglII on CYP125 and BamHI on the vector, allowing expression of the CYP125 gene from a T7lac promoter under isopropyl 1-thio-β-galactopyranoside induction, and producing a recombinant P450 protein with an N-terminal His<sub>6</sub> tag.

Protein was produced in *E. coli* HMS174 (DE3) (typically 15–20 liters, grown in 2×YT medium) by isopropyl 1-thio-β-galactopyranoside (0.15 mM) induction in the presence of the heme precursor 6-aminolevulinic acid (0.1 mM) at OD<sub>600</sub> = 0.6, with temperature then reduced from 37 to 23 °C and culture continued for 24 h. Thereafter, cells were harvested by centrifugation (9,000 × g, 4 °C, 20 min), resuspended in 50 mM potassium phosphate, 250 mM KCl, 10% glycerol, pH 8.0 (buffer A), containing protease inhibitors (Complete EDTA-free protease-free inhibitor tablets, Roche) at 4 °C, and re-centrifuged as before. The pellet was then resuspended in a minimal volume of buffer A (all buffers contained standard protease inhibitors), and the cells were broken by a combination of sonication and French pressure treatment, as described previously (17, 27). The disrupted cell extract was centrifuged (40,000 × g) for 30 min to remove particulate material and then loaded onto a nickel-nitrolotriacetic acid resin column (Qiagen). The column was washed twice in buffer A, containing 30 mM then 75 mM imidazole, and eluted using 200 mM imidazole in the same buffer. The CYP125-containing fractions were pooled and dialyzed versus 50 mM Tris, 1 mM EDTA, pH 7.2 (buffer B), prior to further fractionation using a Resource-Q column on an AKTA purifier (GE Healthcare). CYP125 was bound to the column in buffer B and eluted in a gradient of 0–500 mM KCl in buffer B. The most intensely red CYP125-containing fractions were retained, pooled, and concentrated to a final volume of <1 ml (using a Vivaspin 30 concentrator, Generon) prior to a final gel filtration step using a Sephacryl S-200 column (1.6 × 70 cm) with 10 mM Tris, pH 7.5. CYP125 purity was determined by SDS-PAGE and UV-visible spectroscopy. The most pure fractions were retained, concentrated as previously (to ~500 μM), and used directly for crystallogenesis, or dialyzed into 50 mM potassium phosphate, pH 7.5 (buffer C), containing 50% glycerol and stored at −80 °C.

**Ligand Binding and Thermodynamic Studies**—Optical titrations for determination of azole ligand binding constants (K<sub>d</sub>) values were done as previously described (11). Pure CYP125 (typically 2–5 μM) was suspended in buffer C in a 1-cm path length quartz cuvette and a spectrum for the ligand-free form recorded (250–800 nm) at 25 °C on a Cary UV-50 Bio scanning spectrophotometer (Varian, UK). Azole ligands (clofimazole, econazole, fluconazole, miconazole, ketoconazole, voriconazole, 2-phenylimidazolide, and 4-phenylimidazolide) were titrated from concentrated stocks in dimethyl sulfoxide solvent (apart from the phenylimidazoles, which were prepared in 60% ethanol) until apparent saturation of the optical change was observed. Induced optical change versus ligand concentration data were fitted using Equation 1, which provides the most accurate estimation of K<sub>d</sub> values for the tight binding azole drugs, as we have described in previous studies of the Mtb CYP121 and CYP51B1 P450s (8, 17). Data were fitted using Origin software (OriginLab, Northampton, MA).

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A_{\text{obs}} = \left( A_{\text{max}}/2E_i \right) \times (S + E_i + K_d) - \left( (S + E_i + K_d)^2 - (4 \times S \times E_i) \right)^{0.5} \quad \text{(Eq. 1)}
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In Equation 1, A<sub>obs</sub> is the observed absorbance change at ligand...
Crystal Structure of M. tuberculosis CYP125

data were fitted to the Nernst function (using Origin software) of the solution was measured using a Pt/Calomel electrode (ThermoRussell Ltd.) at 25 °C. The electrode was calibrated to an electrochemical potential of the solution. Other spectral measurements reporting on the sodium dithionite-dependent reduction, binding of CO to the ferrous enzyme form, and nitric oxide to the ferric form (for enzyme quantification and establishment of typical visible spectrophotometer, either aerobically or under anaerobic conditions in a glove box (Belle Technology, Portesham, UK) for ferrous enzymes (8, 28).

CYP125 redox titrations were performed in a Belle Technology glove box under nitrogen atmosphere, as described previously (29). Protein solution (approximately 9 μM in 5 ml of 100 mM potassium phosphate, 10% glycerol, pH 7.0) was titrated electrochemically by the method of Dutton (30) using sodium dithionite as reductant and ferricyanide as oxidant. Mediators were added to facilitate electrical communication between enzyme and electrode (2 μM phenazine methosulfate, 7 μM 2-hydroxy-1,4-naphthoquinone, 0.3 μM methyl viologen, and 1 μM benzyl viologen, to mediate in the range from +100 to −480 mV) (31). Spectra (250−800 nm) were recorded using a Cary UV-Vis Bio UV-visible scanning spectrophotometer. The electrochemical potential of the solution was measured using a Mettler Toledo SevenEasy meter coupled to a Pt/Calomel electrode (ThermoRussell Ltd.) at 25 °C. The electrode was calibrated using the Fe(III)/Fe(II) EDTA couple as a standard (+108 mV). A factor of 244 mV was used to correct relative to the standard hydrogen electrode. Redox titrations were performed in both reductive and oxidative directions to ensure that the redox processes were fully reversible and hysteretic effects were not observed. Absorption change versus applied potential data were fitted to the Nernst function (using Origin software) to derive the midpoint potential for the CYP125 heme iron Fe(III)/Fe(II) couple (29).

Spectroscopic Studies—Electron paramagnetic resonance (EPR) was done on ligand-free and imidazole (10 μM)-bound ferric CYP125 (220 μM) in buffer C. EPR spectra were recorded on a Bruker ER-300D series electromagnet and microwave source interfaced with a Bruker EMX control unit and fitted with an ESR-9 liquid helium flow cryostat (Oxford Instruments), and a dual-mode microwave cavity from Bruker (ER-4116DM). Spectra were recorded at 10 K with a microwave power of 2.08 milliwatts and a modulation amplitude of 10 G. Resonance Raman was done using 15-milliwatt, 406.7 nm radiation from a Coherent Innova 300 krypton ion laser, and acquired using a Renishaw micro-Raman system 1000 spectrophotometer.

CYP125 Crystallization, Structure Elucidation, and Molecular Modeling—CYP125 was concentrated to 13 mg/ml. Sitting drops were prepared by mixing 0.1 μl of CYP125 with 0.1 μl of mother liquor and incubating at 4 °C. Crystallization conditions were refined to two different conditions, both consisting of MgCl₂ with 0.1 mM HEPES, pH 7.0 or 7.5, and PEG 6000 (20%) or PEG 3350 (25%), respectively. The PEG 6000 conditions mainly generated crystals belonging to the C222₁ space group, whereas crystals generated using PEG 3350 belonged to the P2₁2₁2₁ space group. Ligands 4-androstene-3,17-dione (52 mM) and econazole (33 mM) were prepared in ethanol and diluted 1/10 in mother liquor prior to soaking single crystals for 15 min. Single crystals were cooled to 100 K after addition of 10% PEG 200 as cryoprotectant, and data were collected at ESRF and Diamond beamlines. The CYP125 structure was solved by molecular replacement using the P450terp structure as the search model. Full details are in the supplemental data section. Data and final refinement statistics for the CYP125 crystal structures are in supplemental Table S1.

Molecular modeling of the interaction of cholesterol with CYP125 was based on a soft-restrained molecular dynamics (MD) approach previously described for P450s (32). Briefly, cholesterol was positioned in the ligand-free structure of CYP125, close to the positioning of androstenedione in the androstenedione-bound CYP125 structure, in 4 different orientations, so that no steric clashes with CYP125 residues could be observed and such that either the cholesterol tetracyclic moiety or its alkyl chain was pointing to the heme. All 4 positions were chosen so that the cholesterol molecule main axis was aligned with the entrance channel, to minimize the large conformational changes that would occur during the substrate motion in the channel. Up to 5 different dockings were performed from each starting orientation, using small adjustments of the conformation and coordinates. In the following described protocols, the side chains of residues located in a 10-Å sphere centered on cholesterol, as well as water molecules, were defined as the only mobile atoms, to preserve the tertiary structure of CYP125 as observed in the crystal structure. All MD simulations and energy minimization experiments were performed using the NAMD program (33) with Amber force field parameters (34). Topology and parameter files for cholesterol were obtained using the Antechamber program (35) with AM1-BCC charges (36). The cut off parameter for the computation of non-bonded interactions was set to 12 Å, and the electrostatic forces were “softened” by defining a relative dielectric constant of 2 for the system. Energy minimization (1000 steps, conjugate gradient) and MD simulations (200 ps) were initially performed in vacuo at 100 K to thermally equilibrate CYP125-cholesterol complexes. Then, a distance-dependent constraint whose force constant values ranged from 1.5 to 2 (kcal/mol)/Å² was applied comparing the stabilization energy due to the CYP125-cholesterol complexes. Comparison and selection of the docked cholesterol models was done by comparing the stabilization energy due to the CYP125-cholesterol interactions (supplemental Table S4) and the minimal distances between cholesterol heavy atoms and the iron atom of the heme. Minimal distances greater than 7 Å led to the dismissal of the docked model. The model considered for the “Results” and “Discussion” was obtained from a starting position corresponding to orientation C (as represented in Fig. 7).
Reconstitution of Cholesterol Hydroxylase Activity of CYP125—Incubations with CYP125 and cholesterol were carried out in 1 ml of 50 mM potassium phosphate, pH 7.2, using 0.5 μM CYP125, 10 μM *E. coli* flavodoxin, 2.5 μM *E. coli* flavodoxin reductase, 2 nM [3H]cholesterol, and 1 mM NADPH with a NADPH regenerating system (glucose 6-phosphate and glucose-6-phosphate dehydrogenase) (37). The enzymatic reaction was initiated by the addition of NADPH and terminated by vortexing with 2 ml of CH2Cl2. The organic phase was isolated, evaporated, dissolved in acetonitrile, and subjected to HPLC as previously described (37).

To characterize the product of CYP125 activity by gas chromatography-mass spectrometry, the concentration of cholesterol in the enzyme assay was increased to 1 μM. After termination of the enzyme reaction, the substrate and product were extracted, converted into trimethylsilyl ethers, and injected into a VF-35MS capillary column (60 m × 0.32 mm × 0.25 μm) in a splitless mode at an injection temperature of 270 °C with a helium flow of 1.1 ml/min. The initial oven temperature was kept at 200 °C for 1 min, then increased to 280 °C (20 °C/min), ramped up to 310 °C (3 °C/min), and held for 14 min isothermally. The mass spectrometer (Agilent 5973N-MSD combined with an Agilent 6890 GC system) was operated in electron impact ionization (70 eV) at 230 °C. The retention time and mass spectrum of the trimethylsilyl CYP125 product was essentially identical to that of authentic 27-hydroxycholesterol (purchased from Steraloids, Newport RI), with the base peak at m/z 129 and prominent peaks at m/z 417, 456 and 546.

Materials—Bacterial growth medium (Tryptone, yeast extract) was from Melford Laboratories (Ipswich, Suffolk, UK). A 1-kb DNA ladder was from Promega. Azole drugs were from MP Biomedicals Inc. All other reagents were from Sigma and were of the highest grade available.

RESULTS

Genetic Context, Expression, and Production of *M. tuberculosis* CYP125—To define the biochemical and structural characteristics of CYP125, we expressed and purified the P450 from *E. coli*. Purified CYP125 was dark brown (not red) in color, and optical spectroscopy revealed an extensively high spin (HS, >80%) enzyme with heme Soret features at 393 (HS, major) and 416 nm (low spin, LS, shoulder) (Fig. 1A). The HS/LS ratio was affected by temperature, ionic strength, and pH, although the protein was predominantly HS under all conditions. In contrast, and despite apparent homogeneity by SDS-PAGE, certain fractions obtained during gel filtration purification had predominantly LS heme iron with Amax at 415 nm (Fig. 1A). Solvent treatments of HS CYP125 fractions did not result in extraction of potential substrates bound to the enzyme, but did demonstrate that the heme spin state could be readily manipulated by organic solvents (e.g. methanol, see below).

Ligand Binding Characteristics of CYP125—Addition of heme coordinating ligands resulted in occupancy of the 6th (distal) position on the heme iron, with Soret optical shifts seen for imidazole (maximum at 426 nm), cyanide (439 nm), and nitric oxide (433 nm) (Fig. 1B). A fundamental property of P450s is their binding of carbon monoxide (CO) to ferrous heme iron to produce type II (red) shifts of the Soret band. Drugs with other Mtb P450s (e.g. CYP121, CYP51B1, and CYP130) (11, 17, 21). Azoles typically directly coordinate to P450 heme iron to produce type II (red) shifts of the Soret band. For CYP125, unusual binding properties of various azoles were seen. Voriconazole did not induce a spectral shift, whereas fluconazole and ketoconazole produced small type II shifts, suggesting ~20 and 35% heme iron coordination, respectively. In the case of econazole, previous work showed its binding induced a near complete HS conversion (21). Although we found...
for the Fe$^{3+}$/Fe$^{2+}$ transition of the CYP125 heme iron was $-303 \pm 5$ mV (versus NHE), consistent with the mainly HS nature of the P450 (supplemental Fig. S3) (21). Full analyses of EPR, resonance Raman (supplemental Tables S2 and S3), and thermodynamic data are presented in the supplemental data.

Crystallization and Structural Determination of Ligand-free CYP125—In view of the importance of CYP125 to Mtb viability in its host, we determined the crystal structure in both the presence and absence of ligands. The structure was solved to 1.4 Å by molecular replacement using the structure of the *Pseudomonas* sp. P450terp (CYP108A1) as the search model (38). CYP125 has a typical P450-fold with the heme cofactor sandwiched between a major $\alpha$ helical domain and a smaller domain with substantial $\beta$ sheet content (Fig. 3A). An entrance to the active site is clearly defined by the B’ and F $\alpha$-helices and their preceding loop regions (Val$^{96}$–Lei$^{117}$ and Met$^{200}$–Ile$^{221}$, respectively) in addition to contributions by the I-helix (Phe$^{260}$–Thr$^{277}$) and Trp$^{414}$–Leu$^{415}$ from the C-terminal loop region. The entire cavity is lined by hydrophobic residues and resembles a “letterbox” shape with the B’ and F helices defining the opposite sides (Fig. 3B). This putative substrate binding pocket becomes a funnel-like shape, with a progressive narrowing of the active site cavity on approach to the heme. The position and nature of the active site residues in the immediate vicinity of the active group bear remarkable resemblance to the P450terp structure, despite the apparent lack of $\alpha$-terpineol binding to CYP125.

A distinct crystal form (form 2) could be obtained that gave data until 1.7 Å and also contained one CYP125 monomer in the asymmetric unit. No significant changes were observed when comparing both crystal structures (Fig. 3A) with the notable exception of the environment and position of the I-helix residue Val$^{267}$ that is located in the immediate vicinity of the heme distal pocket. In both crystal structures, the Val$^{267}$ side chain is clearly defined as occupying two positions, but the relative occupancy of these positions is markedly different in both crystal structures (Fig. 3C). In one orientation (A), the Val$^{267}$ carbonyl backbone oxygen is involved in I-helix H-bonding interactions, whereas the second orientation (B) positions this

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**FIGURE 2. Optical properties of CYP125 complexes with azoles and cholesterol.** A, spectra for HS CYP125 (solid line) and its type I (dotted line) and type II (dashed line, post-methanol treatment) complexes with econazole. Spectral maxima are at 393, 393, and 418 nm, respectively. B, titration of CYP125 with miconazole, showing a type II optical shift from 393 (solid line) to 421 nm (dashed line) ($K_d = 4.6 \pm 0.4 \mu M$). Intermediate spectra are shown by thin dotted lines. C, type I (substrate-like) spectral shift on addition of cholesterol to CYP125 (from 415 to 393 nm). The starting (mainly LS) solvent-treated form of CYP125 (solid line) and the final HS form (dotted line) at the end of the cholesterol titration are shown, with intermediate spectra as dotted lines. CYP125 concentration in all cases was 3 $\mu M$.
atom within the heme distal pocket. In conformation B, a water molecule occupies a position similar to that observed for the Val267 carbonyl backbone oxygen in conformation A. The relative occupancy of states A and B appears directly linked to the coordination state of the heme iron, with the Val267 A orientation linked to a hexa-coordinate LS state, whereas the B conformation gives rise to a penta-coordinate HS state. In state B, an indirect H-bonding interaction between the Val267 carbonyl backbone oxygen and the water molecule closest to the heme iron is observed. This could account for the observed link between heme iron coordination state and Val267 conformation, as reorientation of this residue affects the heme distal pocket H-bonding network and hence the extent to which water will ligate the heme. Thus, it is possible that upon substrate binding there is a reconfiguration of active site organization and that the structural rearrangement of Val267 is a trigger for aqua ligand displacement and concomitant P450 heme LS to HS conversion. This would link the conserved Thr272 (implicated in proton delivery) via the newly introduced water molecule (only observed in conformation B) to a network of hydrophilic residues (Thr201 and Glu271) and water molecules that could easily serve as a proton relay. It is also likely that CYP125 reduction itself is gated by a LS to HS transition, as seen for other P450s (39, 40).

Crystal Structures of CY125 Androstenedione and Econazole Complexes—Soaking CYP125 crystals with both the steroid androstenedione and theazole econazole produced complexes that were solved to resolutions of 2.0 and 2.2 Å, respectively. In both cases, these molecules are bound within the observed letterbox cavity, with neither ligand able to penetrate the funnel-shaped access tunnel to the heme group (the closest atoms to the heme iron are at 12.9 and 9.3 Å for androstenedione and econazole, respectively). The binding mode for androstenedione (which lacks the alkyl side chain found in cholesterol) is not compatible with P450 oxidation, and the funnel-like nature of the active site clearly prevents the steroid moiety from reaching the direct vicinity of the heme iron (Fig. 4A). Binding of this ligand appears to introduce little change in the protein structure with ligand-protein interactions predominantly through hydrophobic packing of the steroid moiety between residues from the B′-helix and F-helix regions. In addition, a limited set of polar contacts are made between both hydrophilic substituents on the steroid moiety and residues Gly202, Lys214, and Ser217. Econazole binds in a similar hydrophobic region, and is again prevented from further migration into the active site by steric constraints (Fig. 4B). In contrast to androstenedione, econazole binding introduces a minor change in the position and conformation of Val267 due to the close contact made with the econazole chloride substituent that is closest to the heme. In similar fashion to the androstenedione-CYP125 structure, protein-ligand contacts are dominated by a series of hydrophobic interactions with the B′- and F-helix residues, in addition to a single polar contact between the azole moiety and Asp108.
Ligand binding studies revealed the ability of econazole to coordinate heme iron only in an enzyme form obtained by solvent treatment at low temperature, and these data are consistent with conformational rearrangements of the enzyme induced by alteration of the chemical environment and ambient temperature, and that enable the ingress of econazole toward the heme in a proportion of the enzyme molecules.

In addition to androstenedione and econazole, we sought to establish the binding mode of cholesterol to CYP125. However, crystal soaks with cholesterol persistently failed to reveal interpretable density for the cholesterol ligand, whereas co-crystallization attempts failed to generate crystals of suitable quality for diffraction studies. For this reason, we investigated the cholesterol docking mode using molecular modeling methods.

**Molecular Modeling of Cholesterol Binding to CY125A1**—Cholesterol was docked using soft restrained dynamics docking (32) into the CYP125 active site, using the androstenedione binding pocket as the access channel. Several orientations were used as a starting point for docking (Fig. 5), with either the alcohol function on the tetracyclic moiety or the alkyl chain pointing to the heme. During molecular dynamics the backbone CYP125 coordinates were restrained to the conformation observed in the crystal structure. As described in the supplemental data, the final model was chosen considering the highest energy stabilization of the CYP125-cholesterol complex as well as the cholesterol-iron distances. The final model (Fig. 6A) exhibited the greatest stabilization energy among all the models obtained (more than 6 times higher than any others, see supplemental Table S4). The cholesterol is deeply buried in the CYP125 active site, with a calculated buried surface of 312 Å², which corresponds to 86% of the total substrate surface. The tetracyclic portion of the cholesterol occupies the same region of the active site as seen in the androstenedione complex, but the molecule is “flipped” through 180° such that the hydroxyl group on ring A (a carbonyl in androstenedione) is orientated toward the mouth of the active site rather than being internalized. The cholesterol molecule is thus in a conformation where the alkyl chain extends toward the heme, and with the terminal methyl groups (C26/27) positioned close to the heme iron (at distances of 5.3 and 6.3 Å, respectively). This conformation of cholesterol is favored by stabilizing interactions between the hydrophobic cholesterol and several active site residues (Ile97, Leu117, Val267, Phe316, and Trp414), or residues along the active site access channel (Val111, Phe114, and Phe260). Despite the distinct binding modes, the cholesterol-CYP125 model obtained fits well with the CYP125-androstenedione crystal
structure (Fig. 6B), as the tetracyclic portions of cholesterol and androstenedione can be readily superimposed, with methyl groups on the rings oriented in the same direction. The apparent rotation of the tetracyclic moiety between the androstenedione complex and the cholesterol model structures can be explained by the additional favorable binding energy associated with the burial of the cholesterol alkyl chain in the hydrophobic region leading to the heme (as opposed to burial and desolvation of the cholesterol alcohol when considering an androstenedione-like orientation). It is interesting to note that the terminal portion of the cholesterol side chain is in close contact with Val267, an interaction that may be important to promote conformational readjustment of the side chain to displace the distal water and trigger catalysis.

**Experimental Validation of Cholesterol C27 Oxidation by CYP125**—To establish that Mtb CYP125 actually catalyzed oxidation of cholesterol and determine the position(s) of oxidation, we reconstituted the P450 with a bacterial redox partner system (E. coli flavodoxin reductase and flavodoxin proteins and NADPH reductant) that has been well characterized and used widely to drive both prokaryotic and eukaryotic P450 catalysis (41, 42). Experiments were done using gas chromatography-mass spectrometry as performed previously for human CYP46A1 and as detailed under “Experimental Procedures” (37). A single product was formed using the E. coli redox system with CYP125. By comparison with authentic standards, this was shown to be 27-hydroxycholesterol, consistent with our predictions based on structural modeling of the mode of cholesterol association with CYP125 (Fig. 7).

**DISCUSSION**

The location of CYP125 in a gene cluster conserved from Rhodococcus to Mb suggests a likely role in cholesterol metabolism (19). Cholesterol may be important for Mb entry into macrophages, and for establishing infection. The fact that CYP125 is both induced in macrophages and reported as essential for establishing mouse infection is also indicative of a crucial role for this P450 (22, 23). CYP125 is retained in all Mb strains and in some related actinobacteria, e.g. Nocardia and Streptomyces spp. The genetic context of CYP125 is conserved within these bacteria, and the surrounding acyl-CoA dehydrogenase genes (FADE28, FADE29, and FADA5, likely involved in lipid degradation) form an operon with CYP125. Gene knock-out studies on the CYP125 and associated FAD-containing intergenic region (igr) implicated this cluster of genes to have an important role in early mycobacterial infection (43). Despite genetic conservation in non-pathogens, many of the genes within the cholesterol operon are critical for Mb pathogenesis. The Mb cholesterol catabolic gene cluster is under the control of a TetR transcriptional repressor ktsR (Rv3574) likely to have an essential role in pathogenesis and lipid degradation. Genes in this cluster may metabolize diverse lipids, using the mce4 system involved in cholesterol/steroid uptake (44). Collectively these genetic studies and the presence of CYP125 in the cholesterol operon suggest a critical role in bacterial cholesterol metabolism, and in mycobacterial infection and pathogenesis. Our determination of the structure of CYP125 represents the first insight into active site architecture of this important P450, and explains unusual spectroscopic phenomena previously described (21).

Although type II azole binding has been demonstrated clearly for Mtb CYP51B1, CYP121, and CYP130 (11, 12, 16, 17), peculiar type I binding of econazole was reported for CYP125 (21). For the purified, HS form of CYP125 characterized here, this was shown to be the case for econazole. Moreover, clotrimazole and miconazole gave type I binding at low ligand concentrations, but type II binding (heme coordination) at higher concentrations. The phenomena observed for clotrimazole and miconazole suggest alternative binding modes and/or distinct conformers of the P450. On treatment of CYP125 with alcohol (10%) at 10 °C, we were able to produce a mixed spin species that gave type II binding with econazole. Higher concentrations of alcohol destabilized the protein, but also resulted in a further shift toward LS for the ligand-free enzyme. The crystal structure of the econazole-bound (Fig. 4B) CYP125 reveals narrowing of the active site “funnel” precluding further entry of econazole to coordinate the heme iron. The spectral studies are thus suggestive of different conformational states of the enzyme that are favored under different environmental conditions. EPR studies also suggest some heterogeneity in the thiolate-coordinated CYP125 species, which again may suggest the presence of different conformers in the enzyme population studied.

Both crystal structures of the ligand-free CYP125 reveal a clear active site crevice that is roughly rectangular in form and of dimensions well suited to the binding of cholesterol. The majority of this binding pocket is defined by the B' and F helices, which, together with a section of the C-terminal loop and I-helix residues, also contribute to formation of the heme distal pocket. There are some important parallels in relation to the recently determined crystal structure of human CYP46A1, a cholesterol 24-hydroxylase (45; Protein Data Bank code 2Q9F) and of the vitamin D3-bound CYP2R1 (46) (PDB code 3C6G). An overlay of CYP125, CYP46A1, and CYP2R1 reveals that CYP125 and CYP2R1 share a common substrate binding pocket, whereas the sterol moiety of cholesterol in CYP46A1 is bound by a distinct region of the protein (Fig. 6C). In the cholesterol sulfate-CYP46A1 complex, the ligand C24 and C25 carbons are placed closest to the heme iron (both at distances of ~5.7 Å), consistent with the preferred position of oxidation at C24, with the terminal methyl groups more distant. Similarly, the vitamin D3-CYP2R1 complex reveals the C25 and C26/27 carbons located at distances of 5.5 and 6.3 Å, respectively, from the heme iron, which again is in agreement with the observed oxidation at C25 (46). The cholesterol-CYP125 model predicts the C26/C27 cholesterol carbons to be close to the iron center, at a distance of ~5.3 and ~6.3 Å, and we therefore predicted that CYP125 would catalyze oxidation of cholesterol on one or both of the terminal methyl groups. This was proven to be the case in turnover studies, with CYP125 shown to form exclusively 27-hydroxycholesterol.

**CONCLUSIONS**

The CYP125 cytochrome P450 from M. tuberculosis was expressed, isolated, and structurally resolved. The P450 exhib-
its an obvious letterbox substrate access channel of dimensions appropriate for entry of the prospective substrate cholesterol. Complexes with androstenedione and econazole revealed ligand binding near the top of the active site cavity and exclusion for further ingress due to the narrowing of the active site funnel. Although solution state studies reveal econazole (and other azole drugs) are able to coordinate the heme iron under certain conditions, CYP125 clearly demonstrates lower type II binding affinity for a number of azole drugs compared with other Mtb P450s, e.g. CYP121 (17), consistent with the constricted nature of its heme access channel. Our model for the cholesterol-CYP125 interaction, and hence the catalytic activity, was obtained a priori and used to guide further experiments. This model indicates that the alkyl chain of this substrate can extend down the narrow binding funnel with the terminal methyl carbons of the chain presented to the heme iron to facilitate C27 oxidation, as confirmed by turnover studies. Given the likely role of CYP125 in catabolism of host cholesterol, this reaction is likely a primary event that enables the breakdown of the cholesterol side chain. However, the hydroxylation of cholesterol at the terminal position also has the potential to generate a product capable of modulating host
cholesterol synthesis, competitively antagonizing estrogen receptor action, and inhibiting expression of nitric-oxide synthase (1). In this respect, it is tempting to speculate that CYP125 participates in cholesterol oxidation to generate a product that is further broken down to generate metabolic fuel for Mtb and/or is used directly to modulate host responses and thus facilitate persistence of the pathogen.

REFERENCES

1. Umetani, M., Domoto, H., Gormley, A. K., Yuhanna, I. S., Cummins, C. L., Javitt, N. B., Korach, K. S., Shaul, P. W., Mangelsdorf, D. J. et al. (2007) Nature Med. 13, 1185–1192
2. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Ewigmeier, K., Gas, S., Barry, C. E., 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sutliffe, L. J., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) Nature 393, 537–544
3. Fleischmann, R. D., Alland, D., Eisen, J. A., Lathigra, R., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sutliffe, L. J., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) Nature 393, 537–544
4. McLean, K. J., Dunford, A. J., Neeli, R., Driscoll, M. D., and Munro, A. W. (2007) Arch. Biochem. Biophys. 464, 228–240
5. Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003) Mol. Microbiol. 48, 77–84
6. McLean, K. J., Carroll, P., Lewis, D. G., Dunford, A. J., Seward, H. E., Neeli, R., Cheesman, M. R., Marsollier, L., Douglas, P., Smith, W. E., Rosenkrands, I., Cole, S. T., Leys, D., Parish, T., and Munro, A. W. (2008) J. Biol. Chem. 283, 33406–33416
7. Belin, P., Le Du, M. H., Fielding, A., Lequin, O., Jacquet, M., Charbonnier, J., Lecoq, A., Thai, R., Courçon, M., Masson, C., Dugave, C., Genet, R., Pernodet, J. L., and Gondry, M. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 7426–7431
8. Holsclaw, C. M., Sogi, K. M., Gilmore, S. A., Schelle, M. W., Leavell, M. D., Bertozzi, C. R., and Leary, J. A. (2008) ACS Chem. Biol. 3, 619–624
9. McLean, K. J., Warman, A. J., Seward, H. E., Marshall, K. R., Girvan, H. M., Cheesman, M. R., Waterman, M. D., and Munro, A. W. (2006) Biochemistry 45, 8427–8443
10. Charles, M. D., Spellmeyer, D. C., Fox, T., Caldwell, J. W., and Kollman, P. A. (1995) J. Am. Chem. Soc. 117, 5179–5197
11. Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tkatchorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kalé, L., and Schulten, K. (2005) J. Comput. Chem. 26, 1781–1802
12. Cornell, W. D., Cieplak, P., Bayly, C. I., Gould, I. R., Merz, K. M., Ferguson, D. M., Spellmeyer, D. C., Fox, T., Caldwell, J. W., and Kollman, P. A. (1996) J. Am. Chem. Soc. 117, 5179–5197
13. Jakalian, A., Jack, D. B., and Bayly, C. I. (2002) J. Comput. Chem. 23, 1623–1641
14. Wang, J., Chang, R., Wang, W., Gumbart, J., Tkatchorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kalé, L., and Schulten, K. (2005) J. Comput. Chem. 26, 1781–1802
15. Mast, N., Norcross, R., Andersson, U., Shou, M., Nakayama, K., Bjorkhem, I., and Pikuleva, I. A. (2003) Biochemistry 42, 14284–14292
16. Hasemann, C. A., Ravichandran, K. G., Peterson, J. A., and Deisenhofer, J. (1994) J. Mol. Biol. 236, 1169–1185
17. Sligar, S. G., and Gunsalus, I. C. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1078–1082
18. Miles, J. S., Munro, A. W., Rospendorowski, B. N., Smith, W. E., and Kollman, P. A. (1994) Biochemistry 33, 10539–10548
19. Jenkins, C. M., and Waterman, M. R. (1994) J. Biol. Chem. 269, 27401–27408
20. Melser, L., Leadbeater, C., Campopiano, D. J., Baxter, R. L., Daff, S. N., Chapman, S. K., and Munro, A. W. (1998) Eur. J. Biochem. 257, 577–585
21. Chang, J. C., Harik, N. S., Liao, B. P., and Sherman, D. R. (2007) J. Infect. Dis. 196, 788–795
22. Kendal, S. L., Withers, M., Soffair, C. G., Moreland, N. J., Girvan, H. M., Cheesman, M. R., Liddell, D. E., and Munro, A. W. (2007) Drug Metab. Rev. 39, 537–544
23. McIver, L., Leadbeater, C., Campopiano, D. J., Baxter, R. L., Daff, S. N., Chapman, S. K., and Munro, A. W. (2006) J. Biol. Chem. 281, 5099–5108
24. McLean, K. J., Cheesman, M. R., Sutcliffe, M. J., Leys, D., and Munro, A. W. (2006) Trends Microbiol. 14, 220–228
25. Ahmad, Z., Sharma, S., and Katoch, V. M. (2006) Int. J. Antimicrob. Agents 28, 543–544
26. McLean, K. J., and Munro, A. W. (2008) Microb. Drug Resist. 14, 1169–1185
27. Ahmad, Z., Sharma, S., Khuller, G. K., Singh, P., Faujdar, J., and Katoch, V. M. (2006) Int. J. Antimicrob. Agents 28, 543–544