Structural complex of sterol 14α-demethylase (CYP51) with 14α-methylenecyclopropyl-Δ7-24, 25-dihydrolanosterol

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Abstract Sterol 14α-demethylase (CYP51) that catalyzes the removal of the 14α-methyl group from the sterol nucleus is an essential enzyme in sterol biosynthesis, a primary target for clinical and agricultural antifungal azoles and an emerging target for antitrypanosomal chemotherapy. Here, we present the crystal structure of Trypanosoma (T) brucei CYP51 in complex with the substrate analog 14α-methylenecyclopropyl-Δ7-24, 25-dihydrolanosterol (MCP). This sterol binds tightly to all protozoan CYP51s and acts as a competitive inhibitor of F105-containing (plant-like) T. brucei and Leishmania (L) infantum orthologs, but it has a much stronger, mechanism-based inhibitory effect on 1105-containing (animal/fungi-like) T. cruzi CYP51. Depicting substrate orientation in the conserved CYP51 binding cavity, the complex specifies the roles of the contact amino acid residues and sheds new light on CYP51 substrate specificity. It also provides an explanation for the effect of MCP on T. cruzi CYP51. Comparison with the ligand-free and azole-bound structures supports the notion of structural rigidity as the characteristic feature of the CYP51 substrate binding cavity, confirming the enzyme as an excellent candidate for structure-directed design of new drugs, including mechanism-based substrate analog inhibitors.—Hargrove, T. Y., Z. Wawrzak, J. Liu, M. R. Waterman, W. D. Nes, and G. I. Lepesheva. Structural complex of sterol 14α-demethylase (CYP51) with 14α-methylenecyclopropyl-Δ7-24, 25-dihydrolanosterol. J. Lipid Res. 2012. 53: 311–320.

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Sterol 14α-demethylase (CYP51) is a cytochrome P450 (CYP) monooxygenase (EC 1.14.13.70) that catalyzes removal of the 14α-methyl group from cyclized sterol precursors: lanosterol, 24,25-dihydrolanosterol, 24-methylenedihydrolanosterol (eburicol), 29-norlanosterol, and 24-methylene1,29-norlanosterol (obtusifoliol). The CYP51 reaction is a required step in sterol biosynthesis, leading to formation of cholesterol in vertebrates, ergosterol in fungi, and a variety of 24-alkylated ergosterol derivatives in plants and protozoa (1).

Sterols are essential cellular components that generally distinguish eukaryotic membranes (2–5), although some prokaryotes can make them as well (6, 7). Sterols modulate membrane fluidity/permeability and also play multiple regulatory (hormonal) functions related to cell division, growth, and developmental processes. Sterol biosynthesis is an ancient metabolic pathway of prokaryotic origin, the first committed step being squalene epoxidation. The reaction is catalyzed by squalene monooxygenase (EC 1.14.99.7) and produces squalene-2,3-epoxide, which in nonphotosynthetic organisms is then cyclized into lanosterol. It is believed that sterol biosynthesis evolved shortly after molecular oxygen had become available on the planet (8). Presence of molecular oxygen is also a prerequisite for the appearance of monooxygenase activity. CYP51 has been suggested to be an ancestor for cytochrome P450 monooxygenases (9, 10), which now have evolved into the CYP superfamily (>15,000 sequenced genes) catalyzing a vast number of different reactions. In cytochromes P450, the catalytic heme iron is coordinated to a proximal cysteine thiolate. In almost all CYPs, scission of molecular oxygen usually inserts one oxygen atom into...
the substrate and releases one water molecule (11, 12). In addition to molecular oxygen, the reaction consumes two electrons that are delivered to the heme iron from the electron donor partners, such as NADPH-containing cytochrome P450 reductase, and two protons:

$$\text{SH} + 2e^- + O_2 + 2H^+ = \text{SOH} + H_2O$$

The classic P450 catalytic cycle begins from binding of substrate (Fig. 1, step 1). This triggers delivery of the first electron, which reduces the heme iron from its ferric ($Fe^{3+}$) to ferrous ($Fe^{2+}$) state (step 2). Then molecular oxygen binds to the iron on the distal side of its coordination sphere, forming $Fe^{3+}$-OO (step 3). Arrival of the second electron to the ferrous dioxygen complex produces ferric peroxy intermediate ($Fe^{3+}$-O-O$^2^-$) (step 4). Protonation of the distal oxygen forms a ferric hydroperoxo intermediate ($Fe^{3+}$-O-OO$^-$) (step 5), and the second protonation at the distal oxygen atom causes heterolytic scission of the O-O bond and loss of water, generating an iron$^{2+}$ oxo porphyrin cation radical ($Por^+$ =Fe$^{4+}$=O) or Compound I (step 6), which acts as the catalytically active species. At this step, the substrate is oxygenated and the iron returns to its ferric state. High reactivity of Compound I has made it difficult to prove its existence, and it has been successfully captured only recently (13). In the meantime, it was also reported that some P450s can use hydroperoxo (step 5) and peroxy (step 4) intermediate as reactive species, so that the P450 catalytic cycle can be simplified (reviewed in Ref. 14).

Assuming that CYP51 is indeed an ancestral P450, the CYP superfamily evolution, directed at functional diversification and increase in the rate of catalysis to adjust to an ever-changing environment, has involved several other simplifications. Thus, most P450 reactions require only one P450 catalytic cycle. Also, most CYPs display significant substrate promiscuity, and therefore, although they all preserve the basic P450 structural fold, their substrate binding pockets are well known for high structural plasticity, being able to change shape and volume significantly depending on the chemical structure they accommodate. By now the CYP superfamily is so diverse that the heme-coordinated Cys is the only residue conserved in all known sequences. Due to high reactivity of their catalytically active species, many P450s, especially those with broad substrate specificity, are often victims of their own reactions, known as mechanism-based inactivation, by suicide substrates (15). Activated in the P450 reaction, such substrates form a covalent bond with the enzyme, either the apoprotein or the heme prosthetic group (16–19).

CYP51s have also diverged upon evolution: their sequence identity across the biological kingdoms is less than 30%, and yet they all catalyze only one stereospecific reaction. The reaction includes three sequential P450 cycles. The sterol 14α-methyl group is converted into the 14α-alcohol, the 14α-aldehyde, and finally into the formic acid with concomitant insertion of the Δ14-15 double bond into the sterol core. Only then is the product released from the enzyme active site. The three-step reaction is rare among P450s and is only known for CYP11A, CYP17, CYP19 (1), and CYP27A1 (20), all of them metabolizing sterol molecules. CYP51s have strict specificity toward their five natural substrates and do not metabolize any other structures. In vitro, animal and fungal orthologs [CYP51A and CYP51F, respectively, following D. R. Nelson’s nomenclature (http://dnelson.uthsc.edu/CytochromeP450.html)] can demethylate all five sterols; plant CYP51s (CYP51G) appear to be selective to C4-monomethylated obtusifoliol and norlanosterol. The highest intrakingdom diversity in the substrate preferences has been observed in CYP51s from Trypanosomatidae (CYP51E), thus, they share >70% amino acid identity. Thus, the enzyme from Trypanosoma (T) brucei is strictly specific toward C4-monomethylated sterols (21), CYP51 from Leishmania (L) infantum prefers C4-monomethylated sterols but can metabolize lanosterol (22), and T. cruzi CYP51 prefers C4-dimethylated eburicol (23). The differences are largely connected with a single amino acid substitution in the B helix [also known as cytochrome P450 substrate recognition site (SRS) 1 (24)], the plant-specific F105 in T. brucei and L. infantum versus I105 in T. cruzi (animal and fungal CYP51s all having I in this position) (25).

Several attempts to develop substrate-based inhibitors as an alternative to antifungal azoles or cholesterol-lowering drugs have been undertaken but without great success (26–30). Time-dependent inactivation effect was kinetically supported for 14α-ethynyl-lanosterol derivatives (31); however, there has been no follow-up information on their mechanism of action or further use and development.

Recent determination of eukaryotic CYP51 structures (22, 32–34) has shown that, contrary to substrate promiscuous P450s, CYP51s do not display any significant structural rearrangements, either upon azole binding or across species and even kingdoms (35), all having strictly the same set, length, and spatial location of the secondary structural elements [24 helices and 12 β strands separated by 30 loops (33)]. This led us to the suggestion that structural rigidity provides molecular basis for CYP51 functional
conservation (36) and possibly for their elevated susceptibility to azole inhibitors (37). However, in the absence of basic structural information on the substrate-enzyme interaction, the question whether CYP51s experience conformational changes upon sterol binding has remained open. In this study, we determined the structure of T. brucei CYP51 in complex with the substrate analog 14α- methylene cyclopropyl-Δ7-24,25-dihydrolanosterol (MCP). The C4-dimethylated sterol displays higher apparent binding efficiency to the plant-like (F105-containing) CYP51s, but it has a much stronger inhibitory effect on T. cruzi CYP51s, than the 1105F mutant of T. cruzi CYP51s, and labeled with 3H at C3 (21).

Experimental Procedures

CYP51 proteins and their electron donor partner (T. brucei cytochrome P450 reductase) were purified and assayed as previously described (21–23). P450 concentration was determined from reduced CO-binding spectra using coefficient of molar extinction of Δε550–449 = 91.1 cm⁻¹ mol⁻¹ (38). The CYP51 sterol substrates, obtusifoliol and eburicol, were isolated from plant and fungal sources, reducing CO-binding spectra using coefficient of molar extinction described (21–23). P450 concentration was determined from reduction of the CYP51 binding cavity, thus confirming CYP51 as an excellent basis for structure-directed development of new drugs, including suicide substrates, which would be a highly desirable and perhaps more powerful alternative due to increasing severity of the problem of emergingazole resistance in human pathogens.

Synthesis of MCP

The C3-protected C30-aldehyde of lanost-7-en-3β-ol used as a starting material was synthesized from lanost-7-en-3β,30-diol according to published procedures (39, 40). The Δ4-sterol was chosen in preference to the Δ3-isomer because of well-known chemical difficulties related to the Δ3-bond in steroid synthesis of potential mechanism-based inactivators (40). The preparation of the methylene cyclopropene ring structure is a modification of our previous efforts to construct the sterol side chain bearing a terminal methylene cyclopropane group at C26/C27 (41, 42). Cyclopropyltriphenylphosphonium bromide (CPB) (368 mg) was added to anhydrous THF (20 ml) followed by drop-wise addition of nBuLi (1.6 M in hexanes, 0.6 ml) at 50°C under nitrogen protection. Yellow color persisted for 2 h, then the C3-protected C30-aldehyde of lanost-7-en-3β-ol (1 in supplementary Fig. 1) 30 mg in anhydrous THF (10 ml) was added. The reaction mixture was refluxed for 2 h, then cooled to room temperature, and THF was removed in vacuo. The sample was loaded on a gravity chromatography column and eluted with a step-wise gradient of hexane in diethyl ether yielding semi-pure 4,4,14-trimethyl 30, 31-dehydrocholest-7-en-3β-ol (2 in supplementary Fig. 1) in approximately 70% yield. The desired product (~30 mg) was obtained by removal of the C3-protecting group by adding anhydrous diethyl ether (20 ml) followed by addition of lithium aluminum hydride (4 mg) under a stream of nitrogen. The reaction mixture was stirred for 2 h, then water (0.5 ml) was added, the mixture was filtered, and the solvent was removed in vacuo. HPLC (TSK C18 column eluted with acetonitrile/isopropanol, 4/1, flow rate of 2 ml/min operated at room temperature) yielded pure 14α-methylenecyclopropyl-Δ7-24,25-dihydrolanosterol (MCP; Fig. 2A), ~70% yield; RRTc, 2.86; MS: 508.3 (M⁺), 493.3 (M⁺-methyl), 453.4, 395.3, 368.4, 341.2, 300.1, 267.1, 241.0, 207.0, 55.1 (100%); and NMR: 1H: 6.177 (m, 32-H, 1-H), 5.287 (m, 7-H, 1H), 3.223 (m, 3-H, 1H), 0.973 (s, 19-H, 3H), 0.892 (s, 30, 31-H, 6H), 0.869 (d, 26-H, 3H), 0.848 (d, 27-H, 3H), 0.688 (s, 18-H, 3H).

Titration of CYP51s with MCP

Titration of CYP51s with MCP was carried out at 1.8–2 µM P450 concentration in a 50 mM potassium phosphate buffer, pH 7.4, containing 200 mM NaCl and 0.1 mM EDTA. MCP was added to the sample cuvette in the concentration range 0.25–5.0 µM from a 1 mM stock solution in 45% hydroxypropyl-β-cyclodextrin (Cyclodex). The spin state of the P450 samples was estimated from the absolute absorbance spectra (22). The percentage of low-to-high spin transition was calculated from the difference spectra using the extinction coefficient of Δε590–420 = 110 M⁻¹ cm⁻¹. The apparent dissociation constants (Kₐ) of the enzyme-sterol complex were calculated by plotting the absorbance changes in the difference spectra (ΔA₅₉₀₄₂₀) upon titration against free-ligand concentration and fitting the data to a rectangular hyperbola in Sigma plot statistics (21).

CYP51 activity

CYP51 activity was reconstituted as described (21). The standard reaction mixture contained 1 µM CYP51 and 2 µM cytochrome P450 reductase, 50 µM radiolabeled sterol substrate, and varying concentrations of MCP. The reaction was initiated by the addition of 100 µM NADPH and stopped by extraction of the sterols with ethyl acetate. The reaction products were dried, dissolved in methanol, and analyzed by a reverse phase HPLC system (Waters) equipped with a β-R detector (INUS Systems, Inc.) using a Nova Pak C18 column and linear gradient water:acetonitrile: methanol (1:0:4.5:4.5) (solvent A) and methanol (solvent B) from 0 to 100% B for 30 min at a flow rate of 1 ml/min. To monitor possible MCP conversion by CYP51 or release of a CYP51-activated MCP intermediate into the media, 1 µM of CYP51 from T. brucei and from T. cruzi was incubated with 50 µM of radiolabeled MCP (1.88 × 10⁶ dpm/mg) at the standard reaction conditions. Aliquots were taken over time (5, 20, 40, and 60 min), and the sterols were extracted and analyzed as described above.

CYP51 reduction

The ability of CYP51 samples complexes with MCP to be chemically reduced by sodium dithionite and to form complexes with carbon monoxide was confirmed by CO difference spectra. To compare effects of MCP on the enzymatically reduced heme moiety of T. brucei versus T. cruzi CYP51s, CO difference spectra were monitored after 10 min CYP51 incubation in the reconstituted enzyme reaction mixture either with their substrates or with MCP.

Binding to the protein moiety

To test an option of covalent binding of MCP to the protein moiety, 6 nmoles of CYP51, 10 nmoles CPR were incubated with 50 nmoles of radiolabeled MCP (1.88 · 10⁸ dpm/mg) in the reconstituted CYP51 reaction mixture (21) for 2 h, the apoprotein fraction was separated by precipitation with methanol:chloroform:water (2:1:1.5), the lower chloroform phase was mixed with additional methanol, centrifuged for 2 min at 9000 g to pellet protein (43). The protein pellet was dried under a
stream of Ar, dissolved in 0.2 ml of SDS-PAGE sample buffer (8 M Urea, 1% β-mercaptoethanol, 1% SDS, 10% glycerol, 0.015M TRIS, pH 6.8) and heated at 95°C for 3 min. Absence of a covalent bond with the apoprotein was confirmed by 12% SDS-PAGE: the gel was cut into 1 mm strips and the excised bands were solubilized by digestion with 0.8 ml of 50% H2O2 for 24 h at 70°C (44). The digested material was then counted for radioactivity in Scintillation liquid In-Flow ES (IN/US Systems) (1:10) using a Multi-purpose Scintillation Counter LS6500 (Beckman Coulter).

Crystallization

For crystallization purposes, T. brucei CYP51 MCP complex (molar ratio 1:2.5) was concentrated to 250 μM using Amicon Ultra centrifugal filters 50K (Millipore). Crystals were grown in hanging drops by vapor diffusion in 20 mM potassium phosphate solution containing 50 mM potassium phosphate buffer, pH 7.2, 20% polyethylene glycol 3350 at 25°C, cryoprotected with 40% glycerol and frozen in liquid nitrogen.

Structure determination and analysis

The data were collected at the Advanced Photon Source of Argonne National Laboratory (LS-CAT, Beamline 21ID-F) and processed with the XDS package (version January 30, 2009) (45). The structure was determined by molecular replacement using ligand-free T-brucei CYP51 [3g1q] as a search model in Phaser 2.1.4 (46). The model was refined using Refmac5 (47), and the water molecules were placed with ARP/WARP (48). Data collection and refinement statistics can be seen in supplementary Table I. The coordinated and structure factors have been deposited at the RCSB Protein Data Bank under ID code 3P99. Electron density maps are shown in supplementary Fig. II. Structure superpositions were done in LSQkab of the CCP4 suite. Heme-coordinated water molecule from the ligand-free T. brucei CYP51 structure [3g1q] was merged into the T. cruzi CYP51 [3k1o] in Coot. Docking experiments were carried out in Tripos (Sybyl). The figures were prepared in Pymol and Chimera.

RESULTS AND DISCUSSION

Spectral responses of CYP51 to MCP binding

Generally, when a P450 molecule binds substrate, its ferric heme iron (Fe3+) changes its spin state from low (one unpaired electron) to high (five unpaired electrons). This occurs because substrates displace the water molecule from the iron coordination sphere, where in the absence of substrate, the water serves as the sixth (distal) axial ligand. In the absorbance spectra, transition of the iron unpaired electron) to high (five unpaired electrons). This is not always the case with CYP51s: titration with substrates has been known for years to cause only marginal (<20%) low-to-high spin transition in the orthologs from fungi, plant, or mammals (1). Responses of protozoan CYP51s to substrate addition are significantly more generous, ranging from 95% for the T. cruzi ortholog (eburicol) (23) to 50% and even to 90% for the enzymes from T. brucei (21) and L. infantum (22), respectively (29-norlanosterol). However, upon concentration required for crystallization, the high spin form content in the P450 samples drastically decreases, and no density for the sterol molecules is seen in the X-ray structures that we have determined for several crystals obtained from such samples (not shown). So far, MCP (Fig. 2A) has been the only sterol molecule which permits CYP51 concentration without significant loss of the high spin form content.

Spectral responses of four protozoan CYP51 enzymes to MCP are shown in Fig. 2B. Quite peculiarly, although MCP has two methyl groups in the C4 position, it binds with close apparent affinity to all of them. Moreover, spectral responses of the three F105-containing proteins whose preferred substrates are C4-monomethylated [T. brucei, L. infantum, and the I105F mutant of T. cruzi CYP51 (22, 23)] display notably higher amplitudes than that of the T. cruzi ortholog, which corresponds to the 3-, 5- and 6.5-fold higher apparent binding efficiency (ΔA/Kd) relative to T. cruzi CYP51, respectively. The T. brucei CYP51 sample shown in Fig. 2B [84% MCP-bound (high-spin) form] was concentrated and used for cocrystallization. The X-ray structure has been determined with the well-defined electron density for one MCP molecule being present in each of the four P450 monomers in the asymmetric unit, 100% occupancy for molecules A/B and 80% occupancy for molecules C/D (the asymmetric unit can be seen as Fig. 3A).

Inhibitory effect of MCP on CYP51 activity

Inhibition of CYP51 activity was estimated in our standard reconstituted enzymatic reaction containing 1 μM P450, 2 μM CYP51, and 50 μM substrate. Influence on the

![Fig. 2. MCP. (A) Chemical structure (IUPAC carbon numbering). (B) Type 1 spectral responses in different CYP51s. Absolute (upper) and difference (lower) absorbance. P450 concentration, 1.5–20 μM; MCP concentration range, 0.25–5.0 μM. ΔA, spectral response amplitude (% of maximal low-to-high spin transition); Kd, apparent dissociation constant. Relative binding efficiencies (ΔA/Kd) are 1 for the wild-type T. cruzi (I105), 3 for T. brucei, 5 for L. infantum, and 6.5 for I105F T. cruzi CYP51s.](image-url)
MCP is likely to act as a mechanism-based inhibitor, or suicide substrate (see also supplementary Fig. III). HPLC analysis of the sterol fraction after the CYP51 reaction with the radiolabeled MCP as a substrate revealed no MCP metabolite formation (not shown), suggesting that an activated intermediate should have a low partition ratio, forming a covalent bond with the T. cruzi enzyme during the first rounds of its catalysis without being released into the media and thus locking the activity at the first catalytic cycle. This agrees with the data showing no trace of product formation over time after 2 min preincubation of a 10-fold molar excess of MCP prior to addition of the substrate in the reconstituted T. cruzi CYP51 reaction (Table 1).

Because SDS-PAGE analysis of the apoprotein fraction after the CYP51 reaction with MCP revealed no radioactivity covalently attached to the P450 polypeptide band (data not shown), whereas incubation of T. cruzi (but not T. brucei) CYP51 with MCP in the presence of NADPH-cytochrome P450 reductase induced clear loss of the reduced P450 CO-spectra (49–52) (supplementary Fig. IV), we suspect that the covalent bonding is more likely to involve the heme than the protein moiety. Different routes of P450 heme inactivation by suicide substrates have been described, including formation of a heme adduct interacting with the iron atom itself or with the porphyrin ring, such as iron coordinating pyrrol nitrogens, pyrrole carbons, vinyl groups, propionates, or meso-carbons (49, 50, 52–54).

Identification of the exact site of the interaction between T. cruzi CYP51 and the activated MCP intermediate and testing the curative effect of MCP in T. cruzi-infected animals are subjects of a separate study that is currently in progress. Structural analysis of T. brucei CYP51-MCP complex presented here sheds light on the MCP activation mechanism and its selectivity as a T. cruzi CYP51 inhibitor.

**Overall structure of the enzyme-sterol complex**

Binding of MCP does not cause significant conformational rearrangements in the CYP51 backbone, either at the secondary or tertiary structural level. The average root mean square deviation for the positions of the corresponding Ca-atoms in comparison with the ligand-free structure is even lower than the deviation observed upon binding of theazole inhibitor VNI (33) (<0.6 Å versus ~0.7 Å) (Fig. 3B), and it essentially remains within the range of the deviations between the Ca-atom positions in the four CYP51 molecules in the asymmetric unit (Fig. 3C). This agrees with our previous observations (32, 33) and supports the notion about the elevated structural rigidity as a characteristic feature of the CYP51 structure (36, 37). Along the CYP51 polypeptide chain, the regions that display some flexibility are located on the protein surface (the N-terminus, GH-loop, and b3-bundle), whereas the enzyme core, including the substrate binding cavity, represents the least flexible area (Fig. 4). The center of the core is occupied by the heme, which is positioned between the I and L helices, defining the distal and proximal side of the P450 molecule, respectively. Two heme propionates from pyrrole rings A and D maintain the ligand-free T. brucei CYP51 H-bonding (33) with the five amino acid residues (Y103,
The I105F mutant of T. brucei L. infantum T. cruzi CYP51 Conditions

T. cruzi

- No inhibitor (1 μM P450, 2 μM CPR, and 50 μM substrate)
- +MCP 5 μM
- +MCP 10 μM
- +MCP 30 μM

2 min preincubation with 10 μM MCP prior to the substrate addition

| Conditions | Initial Rate | 1 h Reaction |
|------------|--------------|--------------|
| No inhibitor | 100 (3.9) | 100 |
| +MCP 5 μM | 13 | 39 |
| +MCP 10 μM | 5 | 14 |
| +MCP 30 μM | 0 | 4 |

T. cruzi mutant I105F

- No inhibitor (1 μM P450, 2 μM CPR, and 50 μM substrate)
- +MCP 5 μM
- +MCP 10 μM
- +MCP 30 μM

2 min preincubation with 10 μM MCP prior to the substrate addition

| Conditions | Initial Rate | 1 h Reaction |
|------------|--------------|--------------|
| No inhibitor | 100 (4.8) | 100 |
| +MCP 5 μM | 65 | 99 |
| +MCP 10 μM | 46 | 68 |
| +MCP 30 μM | 10 | 44 |

T. brucei

- No inhibitor (1 μM P450, 2 μM CPR, and 50 μM substrate)
- +MCP 5 μM
- +MCP 10 μM
- +MCP 30 μM

2 min preincubation with 10 μM MCP prior to the substrate addition

| Conditions | Initial Rate | 1 h Reaction |
|------------|--------------|--------------|
| No inhibitor | 100 (5.1) | 100 |
| +MCP 5 μM | 74 | 100 |
| +MCP 10 μM | 42 | 91 |
| +MCP 30 μM | 15 | 64 |

L. infantum

- No inhibitor (1 μM P450, 2 μM CPR, and 50 μM substrate)
- +MCP 5 μM
- +MCP 10 μM
- +MCP 30 μM

| Conditions | Initial Rate | 1 h Reaction |
|------------|--------------|--------------|
| No inhibitor | 100 (7.3) | 100 |
| +MCP 5 μM | 55 | 97 |
| +MCP 10 μM | 29 | 53 |
| +MCP 30 μM | 8 | 39 |

\(^a\) V\(_{\text{max}}\) (nmol S/nmol P450/min) at 1μM P450, 2 μM CPR, and 50 μM substrate (eburicol for T. cruzi, obtusifoliol for T. brucei, L. infantum, and the I105F mutant of T. cruzi CYP51s).

Y116, R124, R361, and H420). The iron atom is coordinated to the four pyrrole nitrogens within the heme plane and to the cysteine thiolate (C422) on the proximal side, but its distal axial coordination position becomes vacant as the water molecule present in the ligand-free structure but its distal axial coordination position becomes vacant as the water molecule present in the ligand-free structure (33) is completely expelled from the substrate binding cavity by the 14α-cyclopropyl-group of MCP. This explains the profound low-to-high spin transition of the heme iron induced by MCP in T. brucei CYP51.

MCP binding mode

The substrate binding cavity is formed in front of the distal side of the heme by the B′-helix, B/C loop [P450 SRS1, helices C (CYP51-specific), I (SRS4), loop K/β1-4 (the region preceding SRS5)], and β4 hairpin (SRS6). Together, these secondary structural elements provide 20 sterol-contacting amino acid residues (Fig. 5A), all either conserved across the whole CYP51 family or phyla-specific (Table 2). The substrate access channel entrance (shown with arrows in Fig. 3A and circled in Fig. 3B, C) surrounded by helices A′ ([CYP51/11A/24-specific]), F′ (SRS2), and β4-hairpin is well defined and remains essentially unchanged compared with the ligand-free or azole inhibitor bound structures (Fig. 3D). Because the sterol molecule protrudes deep into the enzyme active site, two of the three channel entrance elements (helices A′ and F′) lose their contacts with MCP, while the β4-hairpin remains connected with the sterol ring A (via van der Waals interactions of M460 and V461 with the α- and β-methyl groups at the C4 atom). The only hydrophilic part of the sterol molecule, the C3β-OH group, is positioned closest to the channel entrance, 3.5 Å from the main chain oxygen of M358 (β1-4).

No other contacts of the C3β-hydroxyl with the protein are seen in the structure (also see supplementary Fig. II-A). The aliphatic arm (C17–C27) is buried in the deepest portion of the cavity between the C-terminal part of the B′/C loop, helix C, and the N-terminus of helix I (supplementary Fig. II-B). Such a deep extension of the substrate binding cavity toward the C-helix is unique for the CYP51 family, reflecting the length of the substrate molecule and the location of the cleavage site (supplementary Fig. II-C).

![Fig. 4. Heme-to-surface gradient of flexibility in the sterol-bound CYP51 molecule. The protein backbone is presented as a cartoon; heme is shown as sticks, MCP is seen as spheres. The C3 oxygen of MCP is red; all other atoms are colored by B-factors that increase from blue to orange. The coloring reveals the three most flexible regions in the complex: the GH-loop, the β3-bundle area, and the N-terminus, all located on the protein surface. The core of the molecule, including substrate binding cavity, displays the lowest flexibility.](image-url)
As expected for catalysis, the α-surface of the sterol molecule is facing the heme plane. This is ~180° rotation compared with 22R-hydroxy-cholesterol in the active site of CYP11A (cholesterol side chain cleavage P450), where, due to its cleavage site position, the sterol core is facing the heme plane with its β-surface (55). Of the 20 sterol contacting residues shown in Fig. 5A, 7 (from Y103 up to Y116 in Table 2) are located in the B′ helix and B′C loop (SRS1), within the CYP51 signature 1 area (1). They encircle essentially the whole sterol skeleton from its β-side. These residues have proved essential for substrate binding in the CYP51s across kingdoms (56), and the structure confirms their role in substrate orientation and maintenance in the catalytically favorable position during the three steps of CYP51 catalysis. Quite opposite to CYP51 signature 1, only two of the residues from CYP51 signature 2 (the middle portion of the I-helix, predicted to serve as SRS4 (57)) are directly involved in the interaction with MCP. Of these, A291 is 3.9 Å from the C30, the catalytic methylenecyclopropyl group which in the CYP51 substrates is attacked by the activated atomic oxygen, whereas T295 (the conserved P450 threonine, 6.1 Å from the heme iron) is only in contact with the methylenecyclopropyl group of MCP, which is the extension of the sterol molecule lacking in the natural CYP51 substrates. Our previous analysis of the ligand-free CYP51 structure suggested that the conserved CYP51 I-helix signature residues, instead of serving as a substrate recognition site, are likely to represent the As expected for the active site cavity, the carbon atoms of MCP are green; the heme is blue. (A) Twenty CYP51 substrate contacting residues (stereoview). Except for M358, only side chains are shown. CYP51 family signature areas I (upper) and II (lower) are depicted as ribbons. (B) Altered side chain locations in the MCP-bound (gold) versus VNI-bound (pink) versus ligand-free CYP51 (cyan). Distances (Å) between the corresponding atoms in the MCP-bound and ligand-free CYP51 structures (dotted line) are shown. The C29 atom of MCP (the β-methyl carbon at C4) is marked with black arrow (C) The 14α-methylenecyclopropyl group of MCP contacts helix I (T295) and loop K/β1-4 (L356), which are presented as ribbons. The C50 atom of MCP (the 14α-methyl carbon) is marked with black arrow.

### TABLE 2. CYP51 substrate contacting residues shown in Fig. 5

| Residues within 4.5Å | Variations | Location | Interaction with the Sterol Molecule |
|----------------------|------------|----------|------------------------------------|
| Y103                 | C          | αB′      | C19; Ring A: C1, C2, C3; Ring C: C11 |
| F105                 | Ph: L(fungi, animals)/I (T. cruzi) | αB′, β′ | C19, Ring A: C29, (4B (3.6 Å)), C3; Ring B: C6 |
| M106                 | Ph: N(plants), T(fungi, animals) | αB′       | Ring B: C6, C7, C8, C9 |
| F110                 | C          | αB′      | C18, Ring D:C16,C17: Arm:C20 |
| V114                 | C          | B′C loop | Arm: C25,C26, C27 |
| A115                 | Ph: V(plants), V/I(fungi), A (animals) | B′C loop | Arm: C25,C24 |
| Y116                 | Ph: F (plants) | B′C loop | C18; Ring C11, C12; Arm C21, C20 |
| M123                 | Ph: N (plants), T (fungi/animals) | αC       | Arm: C27 |
| Q126                 | C          | αC       | Arm: C26, C27,C28 |
| L127                 | Ph: F (plants), K fungi, animals | αC       | Arm: C26, 27,28 (4.4–4.5Å) |
| L130                 | Ph: F (plants), VM,IA (fungi), L/I (animals) | αC       | Arm: C26, 25, 4.9Å |
| M284                 | Ph: L (plants) M/I/L (fungi), M (animals) | αl (N-term) | Arm: C26 (3.4Å) |
| A287                 | Ph: A/G/T (fungi), G (animals) | αl       | Arm: C20,C22,C23,C24,C25,C26; Ring D:C15,C16,C17 |
| F290                 | Ph: M (fungi) L (animals) | αl       | Ring D: C15 |
| A291                 | Ph: G (in some fungi) | αl       | C50 Ring D:C14,C15,C16 |
| T295                 | Ph: S (fungal) | αl       | 14α-methylenecyclopropyl-group |
| L356                 | Ph: I/L (fungi), I (animals) | K/β1-4 loop | 14α-methylenecyclopropyl-group |
| M358                 | Ph: S (fungi), I/T (animals) | K/β1-4 loop | Ring A: C2,C2, C3 |
| M460                 | Ph: M/L (fungi) | β1 hairpin | Ring A: C28(4a),29(4β) (3.9–4.5Å) |
| V461                 | Ph: F/V (fungi) I (animals) | β1 hairpin | Ring A: C28(4a) (4.4Å) |

C, conserved; Ph, phyla-specific; C30, the 14α methyl carbon.
CYP51 proton delivery route (33) that begins at the surface-exposed E205 (helix F, a negatively charged residue conserved in all known CYP51 sequences), proceeds through its salt bridge with H294 [100% conserved, CYP51-specific (1)], and then expands into the hydrogen bond network formed between two ordered water molecules in the I-helix groove, hydroxyl oxygen of T295 (T or S across the CYP51 family) and carbonyl oxygen of A291 (G in some fungal CYP51s), leading to the axial ligand water molecule coordinated to the heme iron (supplementary Fig. V). The enzyme-sterol complex supports this notion, clearly showing that SRS4 in the CYP51 structure is actually located further upstream in the I-helix (residues M284–290 in Table 2). Interestingly, mutation of the corresponding T in rat CYP51 to A, although significantly (6-fold) decreasing enzymatic activity, does not abolish it completely, whereas its substitution to bulkier residues (R/N) makes the activity undetectable (57). This suggests that there is enough space for the catalytic water molecule in this area, the CYP51 I-helix hydrogen bond network can partially compensate for the lack of the threonine hydroxyl in the mechanism of the proton delivery.

**Critical role of F105**

Although the backbone of the CYP51 substrate binding cavity remains very rigid, the side chains of some residues, especially Y103, Y116, and M284 (Fig. 5B), experience quite pronounced repositioning, the MCP molecule pushing them away from the center of the cavity and thus slightly broadening the volume above the heme plane. Though the side chain rearrangements are even more prominent than those seen in the VNI-bound *T. brucei* CYP51 structure, they do not induce loss of the functionally essential H-bonding between Y103/Y116 and the heme propionates (32, 33). Still, positioning of the MCP molecule in the *T. brucei* CYP51 active site seems to be somewhat restricted by F105. This residue does not move much and appears to prevent the sterol molecule from occupying the whole space above the heme that otherwise would now be available for its ring A. The steric hindrance between the sterol ring A and F105 is caused by the MCP C4β-methyl group (C29, marked with an arrow in Fig. 5B) that closely (3.5 Å) approaches the F105 side chain. Due to the F105-C29 contact, the sterol molecule adopts the orientation that brings the 14α-methylenecyclopropyl group of MCP in close contact with helix I (T295, 4.1 Å) and K/β1–4 loop (L356, 3.5 Å) (Fig. 4C), positioning it in such a way that the axial water molecule must be completely expelled from the active site. As a result of intercalation of the MCP 14α-methylenecyclopropyl group between helix I and loop K/β1-4, the 14α-carbon atom appears to be somewhat shifted from the optimal catalytic position (up and to the left in Fig. 5C), forming a 72° angle with the heme plane instead of being located perpendicularly to it, which implies that the orientation of MCP in the *T. brucei* CYP51 active center might differ slightly from the catalytically favorable orientation of the natural CYP51 substrates.

Docking of MCP into the *T. cruzi* CYP51 structure indeed produces an orientation, where the 14α-carbon (C30) is positioned exactly above the iron (Fig. 6). Ring A moves closer toward the B′-helix backbone, and the whole sterol molecule, including the 14α-methylenecyclopropyl group, rotates slightly upward. Interestingly, when MCP adopts this orientation, the former heme-coordinating (axial) water molecule, modeled into the *T. cruzi* CYP51 from the ligand-free *T. brucei* structure, is not expelled from the enzyme active site upon energy minimization [as is observed using the same algorithm for the MCP-*T. brucei* CYP51 complex (not shown)] but only moves about 1.5 Å to the side of the heme toward the I helix forming hydrogen bonds with the hydroxyl oxygen of T295 and carbonyl oxygen of A291, 2.9, and 2.7 Å, respectively. Thus, it is likely, that in *T. cruzi* CYP51 (whose maximal spectral response to MCP is only 41%) I105, which is smaller compared with *T. brucei* F105, does not interfere with the MCP C4β-methyl group, allowing the sterol molecule some repositioning, which results in keeping the axial water in proximity to the heme iron coordination sphere. We surmise this to be crucial for the ability of MCP to act as a *T. cruzi* CYP51 suicide substrate.

**Proton delivery as a possible trigger for MCP activation in *T. cruzi* CYP51**

The fact that CYP51s do not produce 100% high spin form upon substrate binding suggests that in CYP51s the axial water molecule might remain in close proximity to the heme after the substrate binding (33), as has been crystallographically proved for some other CYPs (58, 59), therefore functioning as a shuttle for the delivery of the catalytic protons to the iron-bound oxygen during the three sequential steps of the CYP51 reaction. When MCP binds to *T. cruzi* CYP51, the water presumably remains within the active site (Fig. 6), as it presumably does upon binding of CYP51 natural substrates. Therefore, both iron reduction and oxygen protonation can occur, and
Compound I can be formed. The 14α-methylene double bond of MCP can easily get activated at this step, leading to the opening of the cyclopropyl ring (14), which then forms a covalent bond, presumably with the prosthetic heme group (as suggested by loss of the P450 CO spectra), resulting in the enzyme mechanism-based inactivation.

Vice versa, when MCP binds to the F105-containing T. brucei CYP51, its 14α-methylenecyclopropyl group protrudes closer to the I-helix, and the axial water molecule must be expelled from this area of the binding cavity. We hypothesize that in CYP51s this prevents activation of molecular oxygen by affecting catalytic proton delivery and therefore blocking the P450 catalytic cycle at ferric peroxo intermediate (Fig. 1, step 4). As a result, MCP is not activated and simply acts as a competitive inhibitor, its replacement in the active site by the substrate resuming CYP51 catalytic activity.

In conclusion, in support of the essential role of the axial water in the CYP51 reaction mechanism, the crystallographic data imply that C4-monomethylated analogs of MCP can potentially serve as suicide substrates for F105-containing CYP51s, such as L. infantum and T. brucei orthologs and that further modifications of the MCP-based inhibitory scaffold may amplify inhibitory potency. More generally, the structural information suggests that the F105-based, phyla-specific CYP51 substrate selectivity is connected not with the sterol binding per se but rather with its proper (catalytic) orientation, which is highly tuned for the conserved CYP51 reaction. More F105-containing CYP51s that catalytically resemble the L. infantum ortholog (22) are certain to be found. Overall, the structure of the MCP-CYP51 complex provides new insights into the conserved organization of the CYP51 substrate binding cavity and paves the way for structure-directed development of novel substrate-based CYP51 inhibitors, which can potentially serve as a set of alternative drugs that selectively block sterol biosynthesis in human pathogens.

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