Supplemental data

Substrate Preferences and Catalytic Parameters Determined by Structural Characteristics bf CYP51 from *Leishmania infantum*

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Materials and methods

Chemicals

Glycerol was purchased from MP Biomedical, Triton X-100 was from Roche, ampicillin and DTT from Research Product International Corp., EDTA and methanol (HPLC grade) from Fisher, δ-aminolevulinic acid from Chem-Impex International, CM-Sepharose from Amersham Biosciences, Ni-NTA from Qiagen, acetone from B&J Chrom Pure, acetonitrile (HPLC grade) from ACROS. Fluconazole was from Spectrum Chemical MFG Corp, NADPH from Calbiochem. Other chemicals were purchased from Sigma Aldrich.

CYP51 gene amplification (PCR) conditions

The PCR reaction included 50 ng genomic DNA, 1μM each forward and reverse primers, 0.5 μl FailSafe PCR Enzyme, final volume 25 μl. 25 μl of FailSafe PCR 2x Premix I was added, and amplification was carried out by denaturation at 95ºC for 2 min, then 28 cycles of denaturation at 95 ºC for 60 sec, annealing at 52 ºC for 30 sec and extension at 72 ºC for 140 sec. Terminal extension for 2 min at 72 ºC completed the reaction.

Heterologous expression and purification of L. infantum CYP51

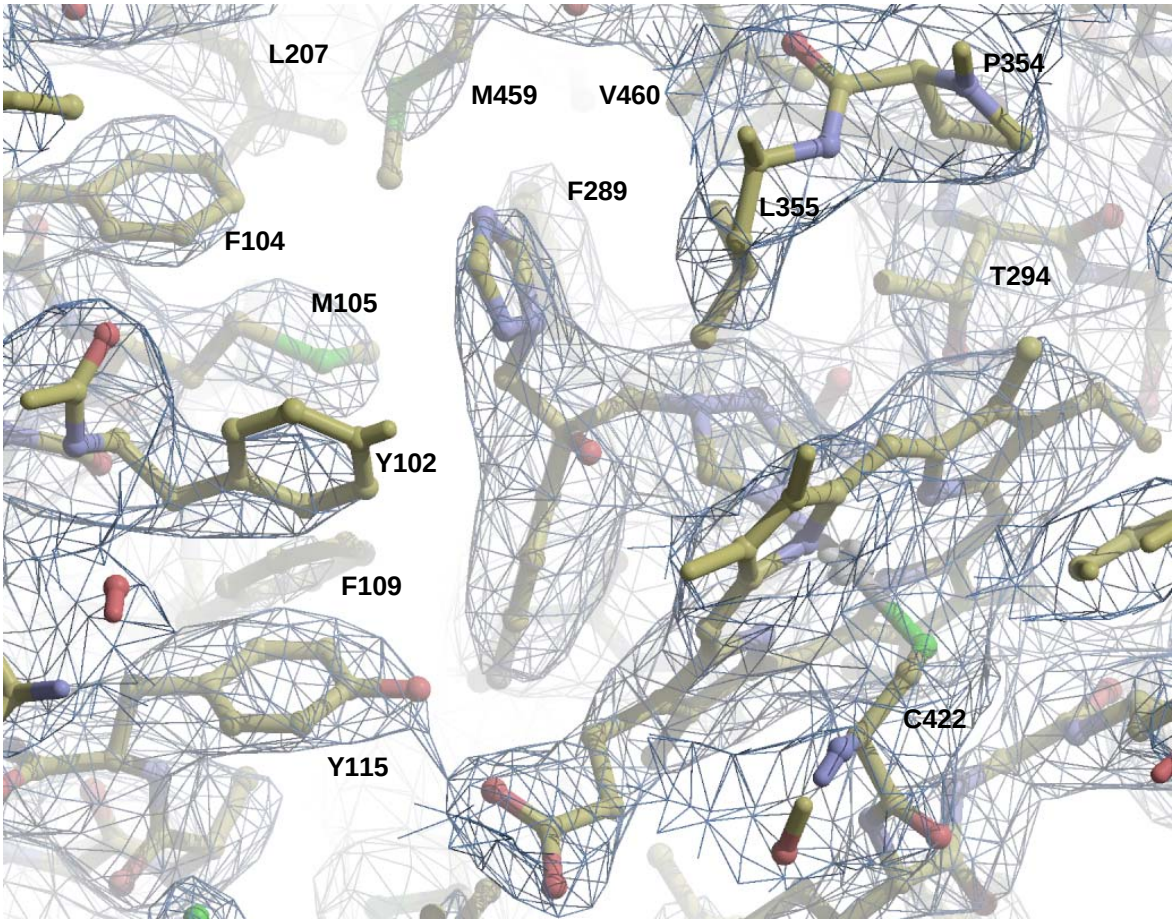
E*. coli* overnight cultures transformed with the *L. infantum* CYP51 plasmid were used to inoculate 500 ml of TB media supplemented with 100 mM potassium phosphate buffer (pH 7.2), 0.1mg/ml ampicillin and 125 μl of rare salt solution* and cultivated at 37 ºC and 250 rpm for 5 hours prior to induction with 1 mM IPTG. Expression was continued at 26 ºC and 200 rpm for 48 hours in the presence of 1 mM δ-aminolevulenic acid. The cells were harvested by centrifugation at 3000 rpm for 15 min (Allegra X-15R Centrifuge, Beckman Coulter). The pellet was resuspended in 100mM Tris-acetate buffer, pH 7.2, containing 1mM EDTA, 0.5mM PMSF and 250 mM sucrose. Lysozyme (1.25 mg/ml) was added, the suspension placed on ice for 10 min and then centrifuged for 20 min at 2700 rpm to collect the membrane

fraction containing *L. infantum* CYP51. The membrane pellet was solubilized with 50 mM potassium phosphate (K-P) buffer, pH 7.2, containing 100 mM NaCl, 0.5 mM EDTA, 10% glycerol (v/v), 0.5 mM PMSF, 1mM DTT and 0.2% Triton X-100 (v/v) (Sol-buffer), sonicated (Sonic Dismembrator model 500, Fisher Scientific), and cell debris were removed by centrifugation (L-80 Ultracentrifuge, Beckman) at 4 ºC, 30,000 rpm for 40 min. The supernatant was applied to a Ni NTA column equilibrated with Sol-buffer without EDTA (Ni-buffer 1). The column was washed with Ni-buffer 1, Ni-buffer 1 containing 500 mM NaCl and 1mM imidazole (Ni-buffer 2) and then with Ni-buffer 2 without Triton X-100. The column was then washed with 20 mM K-P buffer, pH 7.2, containing 500 mM NaCl, 10% glycerol, 0.1mM PMSF, 0.1mM DTT using a 1-20 mM linear gradient of imidazole. The protein was eluted with the same buffer containing 120 mM imidazole. Then it was diluted 10-fold with 20 mM K-P buffer, pH 7.2, containing 10% glycerol, 0.1 mM EDTA, 0.1 mM PMSF and 0.1 mM DTT (CM-buffer) and applied to ion-exchange chromatography on CM-Sepharose equilibrated with CM-buffer containing 50 mM NaCl. The column was washed with 10 bed volumes of CM-buffer using a linear gradient of NaCl (50-200 mM) and *L. infantum* CYP51 was eluted with CM-buffer containing 500 mM NaCl, pooled, concentrated to at least 100 μM (500 μM for the purpose of crystallization), frozen and stored at -80 ºC until use.

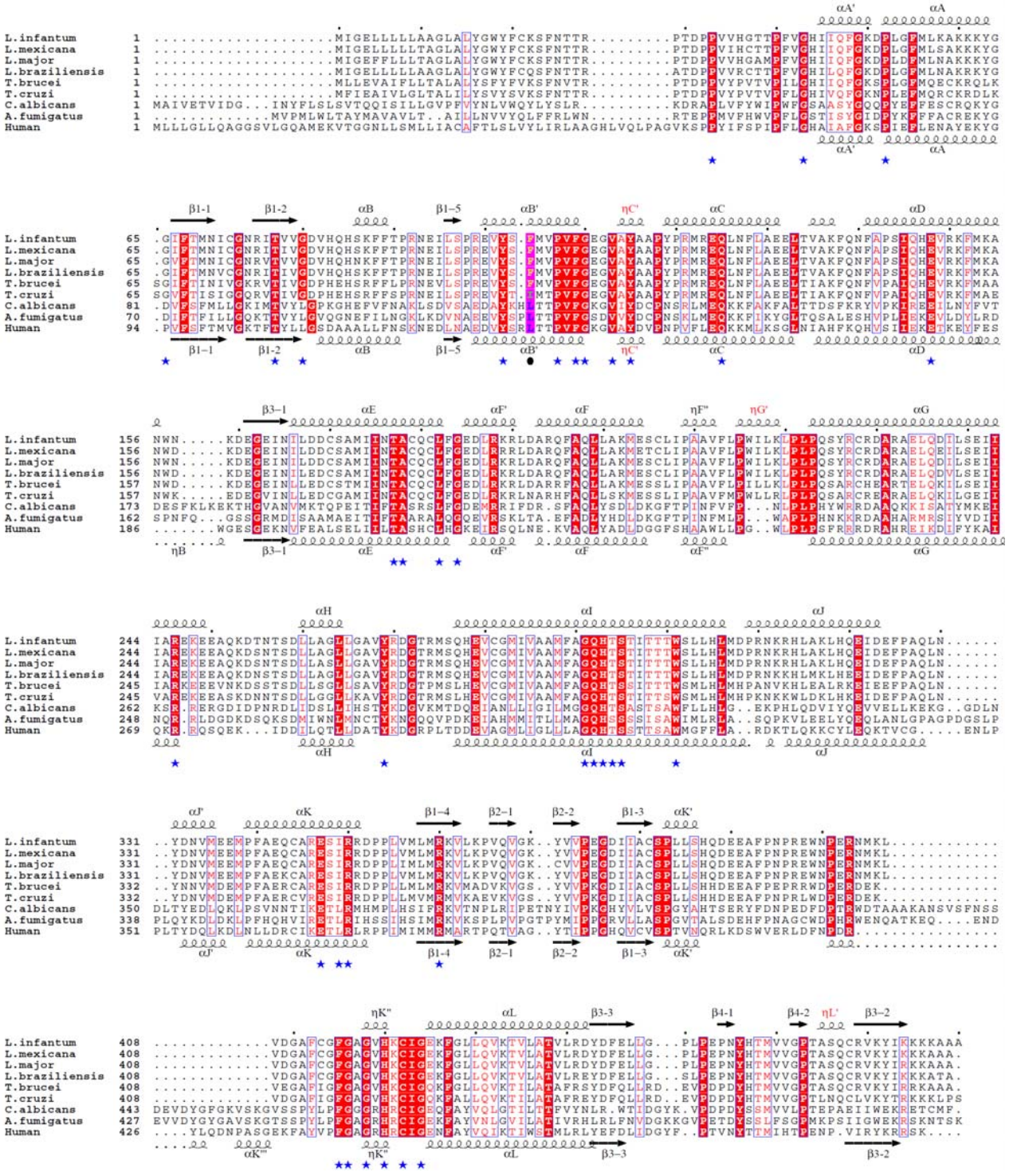
* Rare slat solution : (2.7 g of $FeCl_3 – 6\ H_2O$, 0.2 g of $ZnCl_2 – 4\ H_2O$, 0.2 g of $CoCl_2$ - 6 H_2O, 0.2 g of $NaMoO_4$ - 2 H_2O, 0.1 g of $CaCl_2$ - 2 H_2O, 0.186 g of $CuSO_4$ - 5 H_2O, -.05 g of H_3BO_3 in 90 ml of water, dissolved by addition of concentrated HCl)

Supplemental Table S1. Data collection and refinement statistics

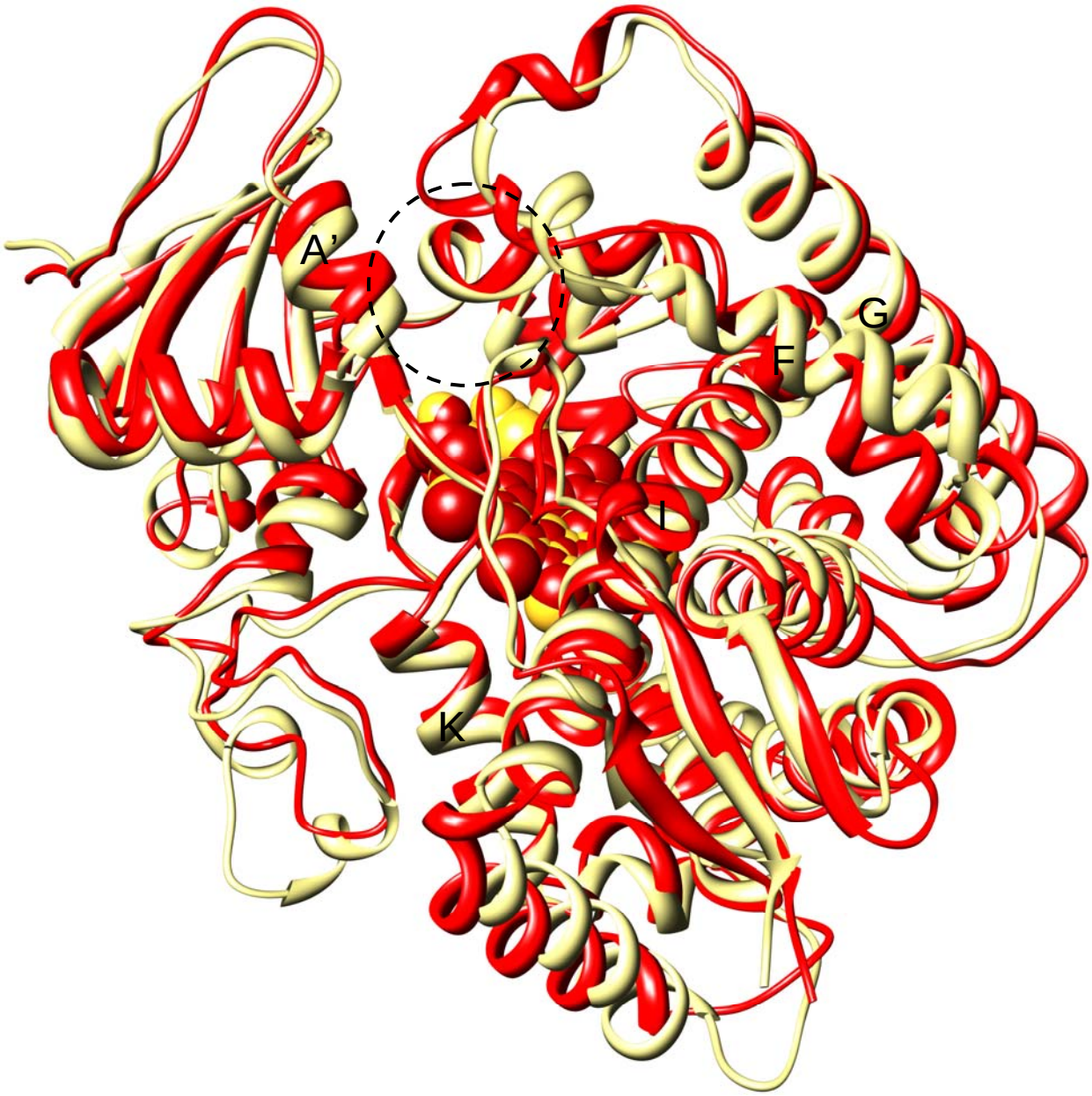
L. infantum CYP51-fluconazole complex (PDB code 3L4D)	
Data collection	
Wavelength, Å	0.980
Space group	P21
Cell dimensions	
a, b, c, Å	84.3, 118.8, 100.9
α, β, γ, °	90 104.8 90
Molecules per asymmetric unit	4
Solvent content, %	49
Resolution (last shell), Å	50-2.74 (2.84-2.74)
R_{merge} (last shell)	0.061 (0.530)
I/σ (last shell)	41.6 (3.2)
Completeness (last shell)	97.9% (94.4)
Redundancy (last shell)	5.6 (5.0)
Refinement	
R-factor	0.219
R-free	0.267
Reflections used	46715
Test set	2495 (5.1%)
Rms deviations from ideal geometry	
Bond lengths, Å	0.003
Bond angles, °	0.6
Ramachandran plot	
Residues in favorable regions	97,2%
Residues in allowed regions	100%
Outliers	0%
Model	
Residues per chain (Average B-factor, $Å^2$)	
protein	447 / 448 / 446 / 444 (56.9)
heme	1 / 1 / 1 / 1 (54.2)
fluconazole	1 / 1 / 1 / 1 (56.5)
water	16 / 11 / 16 / 18 (50.0)
C_8E_5	1 (69.7)



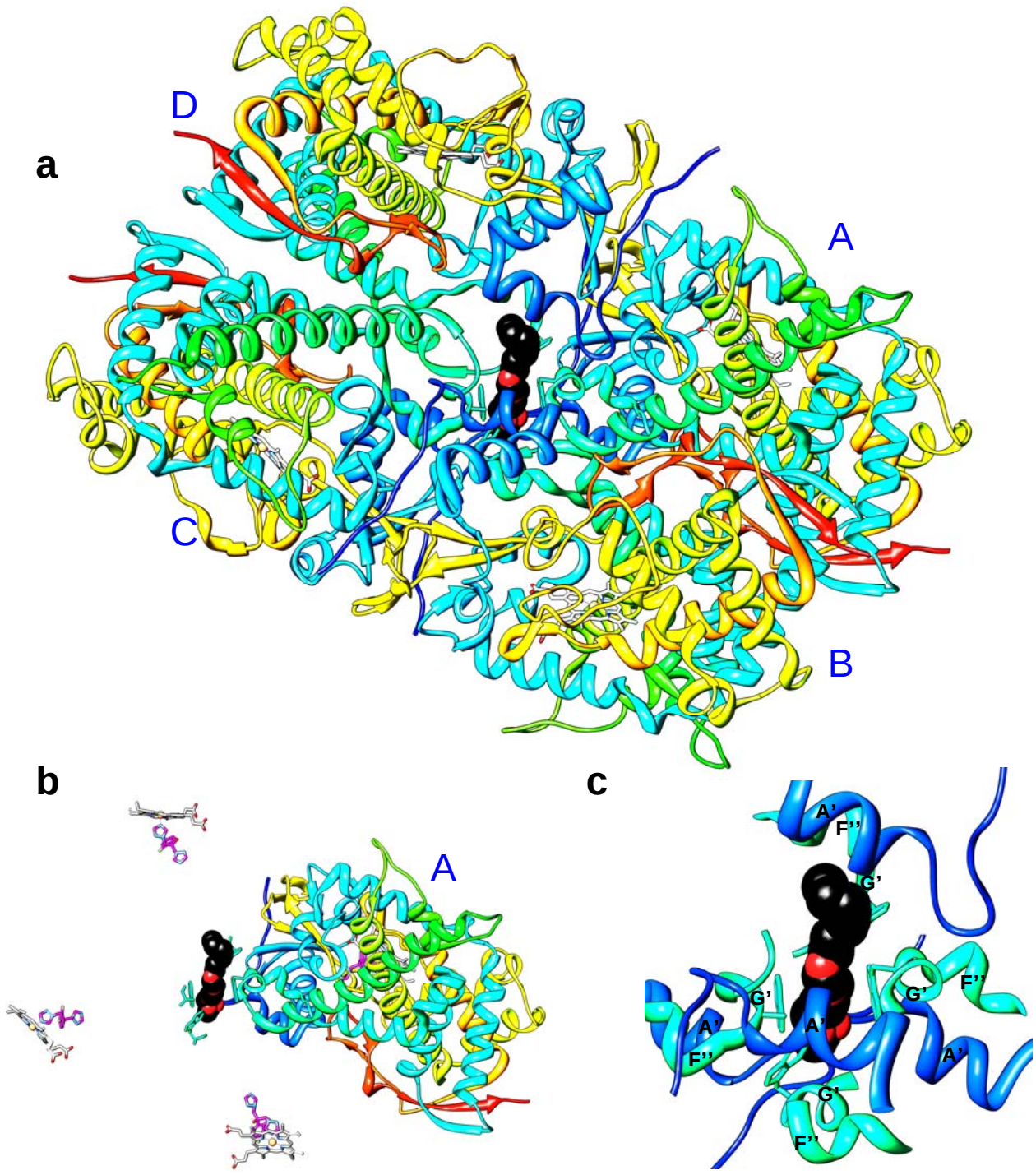
Supplemental Figure S1. 1.5 σ 2Fo-Fc electron density map of the active site area in *L. infantum* CYP51. Fluconazole coordinated to the heme iron is seen in the center.



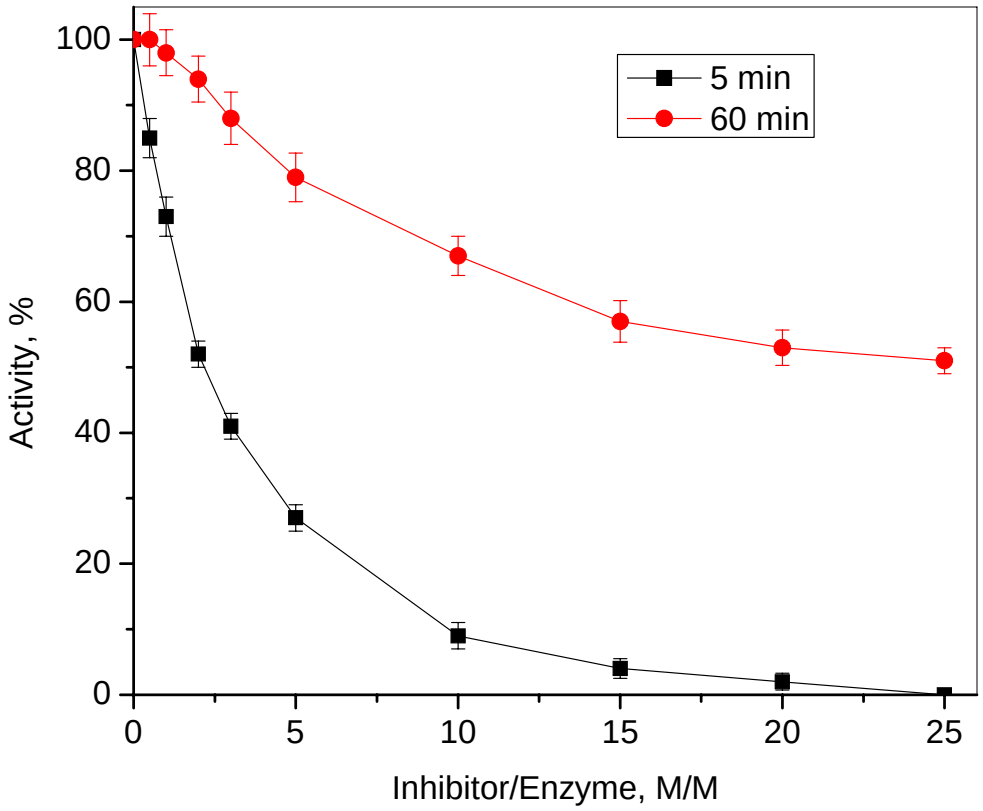
Supplemental Figure S2. CYP51 sequence alignment including four leishmanial proteins, *T. brucei*, *T. cruzi*, two fungal (*C. albicans* and *A. fumigatus*) and human orthologs. The secondary structural elements are from the X-ray structures of: top – *L. infantum* CYP51 [3L4D]; bottom- human CYP51 [3LD6]. The elements specific for CYP51s are indicated in red. The residues conserved across phylogeny are marked with the stars. The black circle marks the phyla-specific (pink framed) residue that defines the CYP51 substrate preferences towards C4-mono (F) vs. C4-doublemethyated (I) sterols.



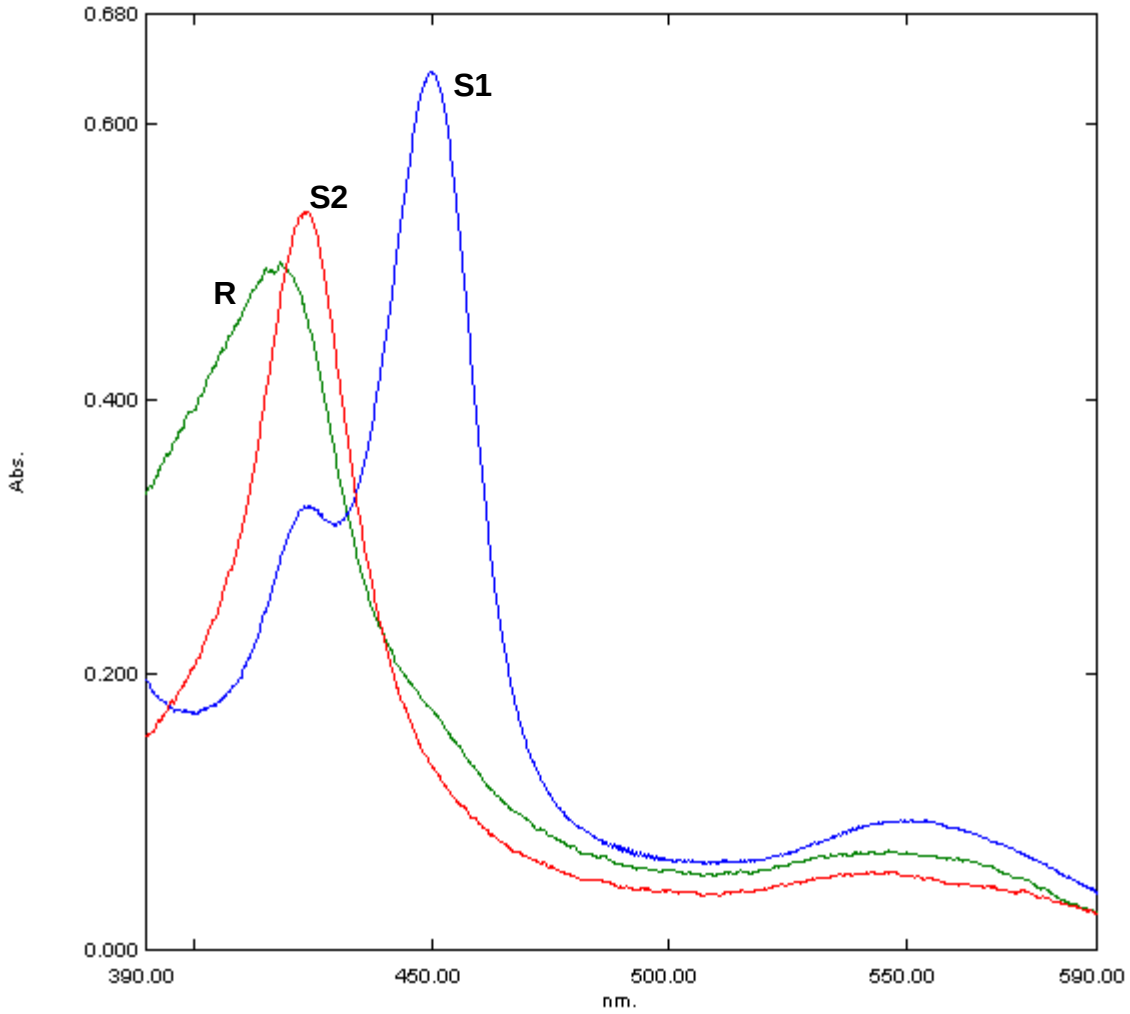
Supplemental Figure S3. Superimposition of the X-ray structures of *L. infantum* (red) and human (yellow) CYP51 orthologs. The protein backbone is displayed as ribbon; the heme is presented as spheres (dark red and gold, respectively); the substrate access channel entrance is outlined with dashed circle. Some helices are marked as reference points.



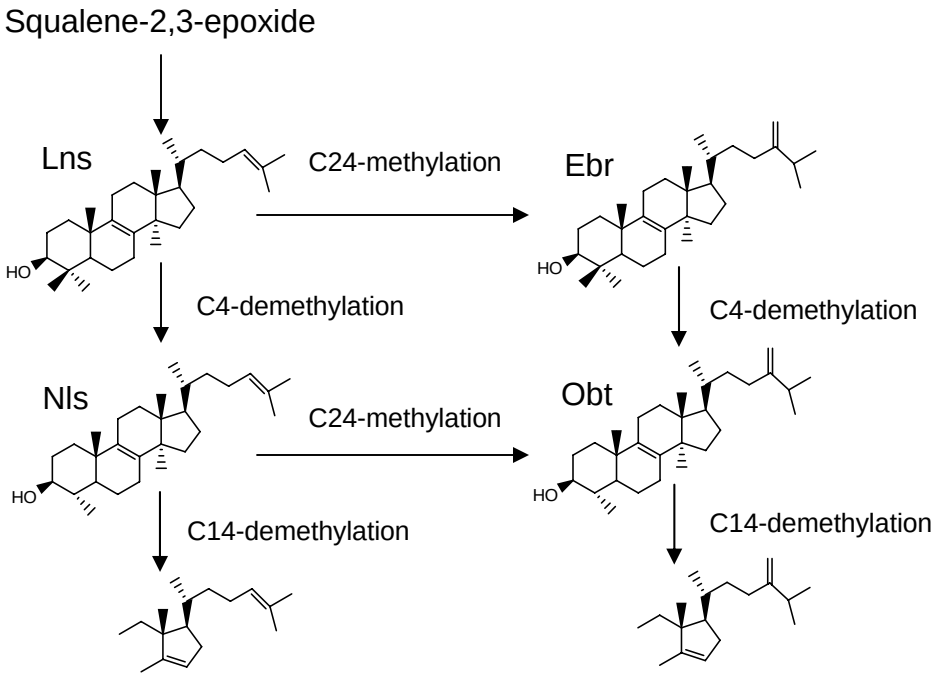
Supplemental Figure S4. **Asymmetric unit in the crystal of *L. infantum* CYP51**. **a**. Four CYP51 molecules (A, B, C and D) are shown as rainbow–colored ribbons (the colors are changing from the blue (N-terminus) to the red (C-terminus). The detergent (C_8E_5) at the interface of the four CYP51 molecules is displayed as spheres with black-colored carbons. **b**. The same orientation with only the ribbon of molecule A shown. Heme is gray, fluconazole is pink. **c**. Enlarged detergent molecule with a fragment of each surrounding protein showing W216 from all four CYP51 molecules as stick models.



Supplemental Figure S5. Inhibition of *L. infantum* CYP51 activity with fluconazole. The details for the CYP51 reaction inhibition were as described in Lepesheva, G. I., Ott, R. D., Hargrove, T. Y., Kleshchenko, Y. Y., Schuster, I., Nes, W. D., Hill, G. C., Villalta, F., and Waterman, M. R. (2007) *Chem Biol* 14, 1283-1293.



Supplemental Figure S6. Absolute absorbance spectra of *L. infantum* CYP51. (S1): initial CO-complex in the presence of obtusifoliol; (**S2**); the spectrum after addition of a 10-fold molar excess of fluconazole to **S1**; (**R**) absorbance of the *L. infantum* CYP51 sample that serves as the reference for the difference CO-spectra (no CO, no fluconazole).



Supplemental Scheme S1. Supported by the *L. infantum* CYP substrate preferences, possible bifurcation of the sterol biosynthetic pathway in Leishmania species