A potential drug target for treatment of Chagas disease, sterol 14α-demethylase from Trypanosoma cruzi (TCCYP51), was found to be catalytically closely related to animal/fungi-like CYP51. Contrary to the ortholog from Trypanosoma brucei (TB), which like plant CYP51 requires C4-monomethylated sterol substrates, TCCYP51 prefers C4-dimethylsterols. Sixty-six CYP51 sequences are known from bacteria to human, their sequence homology ranging from ~25% between phyla to ~80% within a phylum. TC versus TB is the first example of two organisms from the same phylum, in which CYP51s (83% amino acid identity) have such profound differences in substrate specificity. Substitution of animal/fungi-like Ile105 in the B-helix to Phe, the residue found in this position in all plant and the other six CYP51 sequences from Trypanosomatidae, dramatically alters substrate preferences of TCCYP51, converting it into a more plant-like enzyme. The rates of 14α-demethylation of obtusifoliol and its 24-demethyl analog 4α,4α-dimethylcholesta-8,24-dien-3β-ol (norlanosterol) increase 60- and 150-fold, respectively. Turnover of the three 4,4-dimethylated sterol substrates is reduced ~3.5-fold. These catalytic properties correlate with the sterol binding parameters, suggesting that Phe in this position provides necessary interactions with C4-monomethylated substrates, which Ile cannot. The CYP51 substrate preferences imply differences in the post-squalene portion of sterol biosynthesis in TC and TB. The phyla-specific residue can be used to predict preferred substrates of new CYP51 sequences and subsequently for the development of new artificial substrate analogs, which might serve as highly specific inhibitors able to kill human parasites.

Trypanosoma cruzi (TC) is a pathogenic protozoon, the causative agent of Chagas disease, or American trypanosomiasis, threatening lives of more than 100 million people (1). The parasite has a complex life cycle, which involves obligatory passage through four stages, proliferative epimastigote and infective trypomastigote in triatomine insects as vectors, proliferative intracellular amastigotes, and infective bloodstream trypanomastigotes in mammalian hosts (2). The infection is transmitted among humans predominantly by insect vectors or through blood transfusion and primarily affects the heart (chagasic cardiopathy), gastrointestinal tract (megasyndromes), and nervous system (dementia) (3). Left untreated, Chagas disease is commonly fatal. Anti-parasitic therapy is highly toxic and usually ineffective in the chronic stages (1, 4).

Growing knowledge on the basic biology of TC opens new opportunities for rationally developed approaches to treatment of Chagas disease. One of the approaches is to block a key metabolic pathway in the parasite (5). Conserved in eukaryota, sterol biosynthesis is one such target pathway. Either through deoxyxylulose or more generally via the mevalonate pathway (which is likely to take place in Trypanosomatidae (6)), it leads to squalene cyclization in all sterol-synthesizing organisms (www.genome.ad.jp/kegg/pathway/map/map00100.html) and then proceeds through several phyla-specific reactions of sterol core modification, including C4 and C14 demethylation and side-chain modification, resulting in production of cholesterol in animals, ergosterol in fungi, and a variety of 24 alkylated and olefinated sterols in plants and protozoa (7–9). The sterols are essential architectural components of cellular membranes and also fulfill bioregulatory functions, e.g. serving as precursors of steroid hormones and vitamin D in mammals and modulators of growth and development in unicellular organisms (9). Although animals can accumulate cholesterol from the diet, blocking ergosterol production is lethal in fungi, making fungal sterol biosynthesis a very attractive target for treatment of human mycoses (10).

In Trypanosomatidae, structural sterols are found in plasma, inner mitochondrial, and glycosomal membranes (11, 12). Depletion of sterol end products causes trypanosomal cell death as a result of membrane disruption, being especially effective in the exponentially dividing stages of the parasite life cycle (13, 14). Among the genes encoding sterol biosynthetic enzymes in the TC genome (www.tigr.org), sterol 14α-demethylase, as a primary target of clinical antifungal azoles, is of special interest. It is well established that several imidazole and triazole derivatives are highly effective against TC, causing the parasite cell growth inhibition and increasing survival of infected animals (15–17).

Sterol 14α-demethylases are members of the cytochrome P450 superfamily (CYP51), which catalyze oxidative removal of the 14α-methyl group from post-squalene sterol precursors (Fig. 1A). Even with only 22–33% amino acid identity across the biological kingdoms (18), the orthologous enzymes from bacteria to mammals preserve strict catalytic regio- and stereospecificity and have a very limited range of substrates. Until now there were four CYP51 substrates known (Fig. 1B):
CYP51 from Trypanosoma cruzi

FIGURE 1. A, CYP51 reaction. 14α-Methyl group of a sterol molecule is oxidized into an alcohol, then into an aldehyde followed by removal of formic acid and introduction of a Δ14-15 double bond into the sterol core. R1 (angular, 4β-group) is H or CH3, R2 is H or CH2.8, CYP51 substrates. Lanosterol (L), 24,25-dihydrolanosterol (D), and 24-methylenehydrolanosterol (M), physiological substrates of mammalian and fungal sterol 14α-demethylases, are C4-double-methylated; obtusifoliol (O), a physiological substrate of plant CYP51, and 4α,14α-dimethylcholest-8,24-dien-3β-ol (norlanosterol (N)), are C4-monomethylated (equatorial, 4α-group). We have shown in this work that N is metabolized by CYP51 from all biological kingdoms and is likely to be the fifth physiological substrate of sterol 14α-demethylase.

lanosterol (L), 24,25-dihydrolanosterol (D), 24-methylenehydrolanosterol (M), and obtusifoliol (O), with no other compounds reported to be metabolized by this enzyme. Although mammalian and fungal orthologs in vitro are able to 14α-demethylate each of them (although only C4-dimethylsterols are formed in vivo, D/L and M/L, respectively), CYP51 from plants are specific toward their physiological substrate, O (19). We have found recently (20) that, although the pathways leading to obtusifoliol formation in plants and Trypanosomatidae are different, the same profound substrate preference toward this C4-monomethylated sterol exists in CYP51 from Trypanosoma brucei (TB), the cause of African trypanosomiasis.

In contrast, much to our surprise, as described herein, upon initiating studies of TCCYP51 as the target for direct testing of specific inhibitory effects of a broad range of potential anti-chagastic agents in vitro, this enzyme is found to strongly favor substrates having two C4-methyl groups. This is the first case within the same phylum that such profound differences in substrate specificity have been observed. The substrate preferences of TC and TB CYP51 imply that the catalytic sequence after lanosterol formation in TC and TB is different. Although the physiological reason for this at the current level of our knowledge of the biology of the parasites remains unclear, we have found that the molecular basis of the substrate preferences of TCCYP51 is largely associated with a single amino acid in the B′ helix.

MATERIALS AND METHODS

Cloning of TCCYP51—Sequence data for the TCCYP51 gene were obtained from the website of The Institute of Genomic Research (www.tigr.org) using as a template the protein sequence of TBCYP51 (20) for a tblastn homology search. The gene was PCR-amplified from TC genomic DNA using the FailSafe PCR Premix Selection kit (Epicentre). The upstream primer 5′- CGGCAATATGTTTATTGGAAAGCGATGTATTGG-3′ contained a unique NdeI cloning site (underlined) and corresponded to the TCCYP51 cDNA from 1 to 25 bp. The downstream primer 5′- CGCAAGCGTTCAAGTGTAGTGATGCGAGGCAATT-TCTTCTTGCC-3′ included a unique HindIII cloning site (underlined) followed by a stop codon (bold) and C-terminal 4-histidine tag (italics) and was complementary to TCCYP51 sequence from 1443 to 1423 bp. The reaction conditions were the same as described for cloning of TBCYP51 (20), the PCR products were subcloned into pGEM-T Easy vector (Promega), and the correctness of the inserts confirmed by DNA sequencing. The TCCYP51 DNA and protein sequences were deposited into the NCBI data base (accession number AY856083). To obtain the expression construct, the TCCYP51 insert was excised by digestion with NdeI and HindIII (New England Biolabs) and cloned into the pcW expression vector (21). The expression plasmids were sequenced again and transformed into Escherichia coli HMS-174 cells (Novagen).

Site-directed Mutagenesis—The QuickChange mutagenesis kit (Stratagene) was used to destroy the internal NdeI site at 1113 bp (silent mutation Ser371Ser, TCA to TCT), to introduce N-terminal modifications in order to increase the P450 expression level in E. coli (Table 1) and also to mutate Ile105 (ATT) to Phe (TTT) and Thr107 (ACA) to Val (GTA).

Expression in E. coli and Purification—CYP51 from TB, human (HU), Candida albicans (CA), and Mycobacterium tuberculosis (MT) and TB and rat cytochrome P450 reductase were all expressed and purified as described previously (20). TCCYP51 was expressed at conditions similar to the TBCYP51 ortholog and purified using three chromatographic stages, including a nickel-chelating affinity column as described for human CYP51 (22), negative chromatography on Q-Sepharose (Amersham Biosciences), and cation exchange on SP-Sepharose (Amersham Biosciences). Molecular weight and purity of the proteins were confirmed by SDS-PAGE.

Preparation of Sterol Substrates and Synthesis of 14α-Amino-derivatized Substrate Analogs—O, L, D, and M were isolated from plant and fungal sources and labeled with 3H at C3 as described (20). N was from the Nes steroid collection (23) and labeled under the same conditions as other sterols. 4,4-Dimethyl-14α-amino-cholesterol-7-en-3β-ol (AL7) and 4,4-dimethyl-14α-amino-cholesterol-8-en-3β-ol (AL8) were synthesized as shown in Scheme 1 (compounds 5 and 6, respectively).

For routine monitoring of the product profile, steroids were injected into a GC column packed with 3% SE-30 from a Hewlett 5890 Packard Series II operated isothermally at 240 °C. Cholesterol was used as the reference specimen from which the retention times of unknown specimens relative to the retention of cholesterol were calculated. GC-mass spectroscopy was operated using a HP 6890 GC interfaced to a 5973 mass spectrometer at 70 eV. GC was performed using an Agilent HP-5 column (30 m × 25 μm in diameter). Film thickness was 0.25 μm, and the flow rate of He was set at 1.2 ml/min. The temperatures for the
GC to mass spectroscopy interface, mass spectroscopy ion source, and quadrupole were 280, 250, and 230 °C, respectively. Helium at 8 p.s.i. was used as the carrier gas. In this system cholesterol elutes at 13 min. 1H NMR spectra were obtained on a Varian Unity Inova 500 MHz spectrometer at ambient temperature in deuterochloroform with tetramethylsilane as the internal standard.

Spectroscopic Measurements—Absolute and difference absorbance spectra of TCCYP51 were taken at room temperature using a Shimadzu UV-240IPC spectrophotometer. The high spin form content was estimated from the absolute spectra using the ratio (ΔA 393–470/ΔA 417–470) (20). The Na2S2O5-reduced carbon monoxide complex (CO) difference spectra were used to measure the P450 concentration (molar extinction coefficient 91 mM−1 cm−1) to test correspondence between the total amount of hemoprotein (molar extinction coefficient 117 mM−1 cm−1) and P450 complexes and to confirm the absence of conversion into the inactive P420 form. Specific heme content was estimated as the ratio between hemoprotein and total protein determined using the BSA protein assay reagent (Pierce). The abilities of rat and TB cytochrome P450 reductase to support electron transport from NADPH to TCCYP51 were compared as both the half time and efficiency of the enzymatically reduced CO-complex formation at 1 mM stock solution of 45% 2-hydroxypropyl-β-cyclodextrin (Cycloextrin Technologies Development, Inc.) in the range 0.25–20 μM. Fluconazole (ICN Biomedicals, Inc.) and 14α-aminomethyl derivatives of L, AL7 and AL8, were dissolved in Me2SO (2 mM) and added in the range 0.1–20 μM. The corresponding volumes of 2-hydroxypropyl-β-cyclodextrin or Me2SO were added to the reference cuvette. Maximal spectral response per nanomole of P450 (type I, ΔA 390–420 for substrate binding; type II, ΔA 426–410 and ΔA 426–390 for nitrogen coordination for fluconazole and amino derivatives of L, respectively) and apparent dissociation constants were determined as previously described (22) by plotting absorbance changes against the concentration of free ligand and fitting the data to a rectangular hyperbola using Sigma Plot Statistics.

Inhibition of TCCYP51—The inhibitory effects of fluconazole and the two amino-substrate analogs were compared as TCCYP51 activity in the presence of increasing concentrations of the tested inhibitors (1–50 μM) and expressed as the molar ratio 1/P450 at which the activity decreases 2-fold.

Homology Modeling—A molecular model of TCCYP51 was constructed using MTCYP51 (1ea1) (41% structural identity), CYP3A4 (1tqn) (29% identity), and 2C9 (1nr6) (29% identity) as templates. The sequences were aligned, and the coordinates were obtained from the Swiss Institute of Bioinformatics. Coordinates for heme and fluconazole were taken from MTCYP51 (1ea1). Mutations were modeled in Swiss-PDB Viewer.

RESULTS

Cloning, Expression, and Purification of TCCYP51

The sequence of the amplified TCCYP51 gene corresponds to the preliminary DNA sequence in the TIGR data base with the exception of one, silent base pair substitution (G78A). The protein consists of 481 amino acid residues having a molecular mass of ~55 kDa. Although the N-terminal part of the gene is not highly GC-rich (AT/GC ratio is 55/45 for the first 40 codons), the wild type TCCYP51 gene was not expressed in E. coli in the P450 form. Substitution of the second codon to alanine (GCT) (21) led to low P450 expression (Table 1). A further increase of the AT content of the 5′-end by silent mutation in codon 4 and insertion of the substitution E5K allowed us to increase the expression to 250–300 nmol of P450 per liter. Poor interaction of the four His-tagged...
CYP51 from Trypanosoma cruzi

TABLE 1

Impact of N- and C-terminal modification of the TCCYP51 gene on the P450 expression level and purification yield

Amino acid and cDNA sequences (construct 1) are deposited into NCBI (GenBank™ accession number AY856083). The complete amino acid sequence of TCCYP51 is shown in Supplemental Fig. 1. Mutations are underlined. Construct 4 was used as the wild type protein for the work reported.

| Construct | N terminus | C terminus | P450 expression (nmol/l) | Yield after Ni²⁺ column (%) |
|-----------|------------|------------|--------------------------|----------------------------|
| 1         | MFIEA      | 4-His 250–300 CAT-CAC-CAT-CAC-TGA | 5–10 | <15 |
| 2         | MAIEA      | 4-His 250–300 CAT-CAC-CAT-CAC-TGA | 250–300 | <20 |
| 3         | MAIKA      | 4-His 250–300 CAT-CAC-CAT-CAC-TGA | 250–300 | 80–90 |
| 4         | MAIKA      | 6-His 250–300 CAT-CAC-CAT-CAC-CAT-CAC-TGA | 250–300 | 80–90 |

TCCYP51 with the nickel column suggested limited availability of the TCCYP51 C terminus, perhaps due to proline 480 restricting its local flexibility. Insertion of two additional His residues into the TCCYP51 C-terminal His tag solved this problem. The yield of the electrophoretically pure P450 after the three-step purification procedure (Fig. 2A) was about 50%.

Spectral Characteristics

TCCYP51 is purified in the low spin form (Fig. 2B) with a Soret maximum at 417 nm and equal intensities of α-(568 nm) and β-band (536 nm) absorbances, which is typical for sterol 14α-demethylases. The purified protein had a spectrophotometric index of A417/280 1.6 and a specific heme content of 16.6 nmol/mg. The carbon monoxide spectrum of Na2S2O4-reduced TCCYP51 is stable at room temperature for at least 30 min and had a maximum at 448 nm. P420 content does not exceed 5%.

Selection of an Electron Donor

Both rat and TB cytochrome P450 reductase support electron transfer from NADPH to TCCYP51, with maximal efficiency 85 and 100% that of the amount of chemically reduced P450, respectively. The electron transfer, however, at least in vitro conditions, occurred faster with the reductase from TB (Supplemental Fig. 2). The fact that TB cytochrome P450 reductase is a better electron donor for TCCYP51 correlates with our previous data on the reduction of TBCYP51 (20) and implies that the two protozoan CYP51s are likely to have high similarity in the organization of the region of interaction with their electron donor partner.

Substrate Preferences of TCCYP51

Preferences of TB and TC CYP51 for sterol substrates are remarkably different. Although TBCYP51 profoundly prefers O of the four known substrates of sterol 14α-demethylase (20), TCCYP51 demethylates this sterol very poorly (0.06 min⁻¹) (Table 2). Instead, it shows the highest turnover number upon 14α-demethylation of M (2.4 min⁻¹). Metabolism of D upon the same conditions is about 30% slower (1.6 min⁻¹). The rate of 14α-demethylation of L is only 0.57 min⁻¹, although the initial accumulation of the 14α-carboxy aldehyde intermediate from L occurs more than 3-fold faster than the overall demethylation reaction (1.7 min⁻¹). The tendency of TCCYP51 to form the aldehyde intermediate from L resembles that observed with TBCYP51, although the ratio between the rates of formation of the intermediate and the product from L by TBCYP51 is much higher, reaching about 80 (20). Substrate binding parameters calculated from type 1 spectral responses of TCCYP51 (blue shift from 417 to 393 nm in the Soret band) upon sterol versus 2-hydroxypropyl-β-cyclodextrin addition, including maximal amplitude, which reflects the increase in the high spin content (hs%), apparent dissociation constants (Kₒ), and their ratio (hs/Kₒ), suggest that the differences in the rates of sterol metabolism are connected with the ability of the enzyme to interact with the substrate. Comparison of the values calculated for M, D, and L demonstrates a role for the geometry of the hydrophobic side chain. Both M and D have a flexible side chain, but the larger side chain size caused by the additional 24-methylene group of M in this region is slightly preferable. Alternatively, a decrease of the side chain flexibility by the addition of a 24(25)-double bond (L) further affects interaction (~2-fold) and product formation (~3-fold), although the rate of production of the aldehyde intermediate remains practically the same. This 2-fold reduction due to the side chain alteration is also observed upon comparison of the binding and metabolism of O and its C24-demethylated analog N (which we tested as a potential substrate for trypanosomal CYP51 because it was listed among the sterols identified in vivo in Leishmania species (25, 26)). However, the major differences in catalytic activity and binding parameters (M, D, and L versus O and N) are derived from the geometry surrounding the sterol C4 atom; both the equatorial (α) and the angular (β) methyl groups are strongly favored by TCCYP51.

Plant-like and Fungi/Animal-like Sterol 14α-Demethylases

It is generally accepted that plant sterol 14α-demethylases differ from the rest of the CYP51 family by being specific toward their physiological substrate O (27–30), although the structural basis for this preference remains unknown. We have found recently that the same strict specificity toward obtusifoliol is demonstrated by TBCYP51 (20). In this study reconstitution of the reaction of 14α-demethylation of the five sterols by mammalian (HU), fungal (CA), bacterial (MT), plant (SB), and TB CYP51 (Fig. 3A) showed that all the enzymes, including plant...
and TB orthologs, can also metabolize N. This indicates that it is the requirement for the absence of the β-methyl group at the sterol C4 atom (not the side-chain composition) that makes plant and TBCYP51 catalytically distinct from the other orthologs. The other tested sterol 14α,15α-dimethylases catalyze 14α-demethylation of all the five sterols, yet the relative rates of their turnovers are different.

TC, CA, and HU CYP51 do not prefer C4 monomethylsterols. Although TCCYP51 is the only example among them, which (still able to metabolize O and N) clearly favors C4 dimethylated substrates and even demonstrates selectivity among them, we consider the TC ortholog as a fungal/animal-like enzyme for two reasons. First, the physiological substrates of mammalian and fungal CYP51 in vivo (L, M, D) are C4-dimethylated, and second, at the reconstitution conditions producing much lower turnovers, HU/rat and CA/yeast sterol 14α-demethylases were reported to express some preferences toward their natural substrates, D and M, respectively (19, 31). Thus, although the average sequence homology between the CYP51s studied here does not reflect the observed functional differences (Fig. 3B) based on their substrate preferences, the orthologs from TB and TC belong to plant-like and fungi/animal-like groups of sterol 14α-demethylase, respectively.

### Table 2

Sterol binding and catalytic activity of TCCYP51

| Substrate | Wild type | 1105F |
|-----------|-----------|-------|
|           | Spectral response |        | Spectral response |        | Turnover | Turnover |
|           | hs\textsubscript{m} | K\textsubscript{d} | hs\textsubscript{m}/K\textsubscript{d} | hs\textsubscript{m} | K\textsubscript{d} | hs\textsubscript{m}/K\textsubscript{d} |       |         |
| N         | 10 ± 0.7 | 9.5 ± 1.4 | 1.2 | 0.03 ± 0.01 | 54 ± 2.2 | 0.04 ± 0.01 | 135 | 4.6 ± 0.5 | 113 | 113 |
| O         | 9 ± 0.6 | 4.4 ± 0.7 | 2.0 | 0.06 ± 0.01 | 45 ± 3.4 | 0.7 ± 0.05 | 64 | 3.6 ± 0.3 | 150 | 150 |
| L         | 21 ± 1.8 | 1.9 ± 0.3 | 11 | 0.57 ± 0.004 | 20 ± 2.1 | 2.3 ± 0.2 | 8.7 | 0.16 ± 0.02 | 1.3 | 1.3 |
| D         | 30 ± 1.7 | 1.2 ± 0.1 | 25 | 1.6 ± 0.1 | 23 ± 2.0 | 1.4 ± 0.2 | 21 | 0.42 ± 0.03 | 1.2 | 1.2 |
| M         | 36 ± 0.9 | 0.8 ± 0.1 | 45 | 2.4 ± 0.1 | 35 ± 2.7 | 0.9 ± 0.1 | 39 | 0.71 ± 0.05 | 1.3 | 1.3 |

### Graphs

**Figure 3. CYP51 from different phyla.**

A. Relative rates of conversion of five sterol substrates. Maximal turnover numbers with the preferred substrate (nmol/nmol P450/min, shown in brackets) for each tested CYP51 ortholog are expressed as 100%. The data represent the mean from four experiments; S.D. do not exceed 10%. B. Percentage of amino acid identity.

**Figure 4.**

**A** and **B**. Relative rates of conversion of five sterol substrates. Maximal turnover numbers with the preferred substrate for each tested CYP51 ortholog are expressed as 100%. The data represent the mean from four experiments; S.D. do not exceed 10%.

**Table 3.**

**Effect of I105F Mutation on Substrate Binding and Catalysis—Alignment of CYP51 from Trypanosoma cruzi with 7 trypanosomal CYP1s with 59 sequenced orthologs from other phyla reveals only two amino acid residues, which are animal/fungi-specific in TB CYP51 (Phe\textsuperscript{105} and Thr\textsuperscript{107}) but plant-specific in TC CYP51 (Ile\textsuperscript{105} and Thr\textsuperscript{107}), both located in the B' helix (Fig. 4A). The B' helix represents the N-terminal part of cytochrome P450 substrate recognition site 1 (32). Alterations in the substrate specificity upon site-directed mutagenesis in substrate recognition site 1 have been reported for many drug-metabolizing P450s (33, 34).

In the molecular model of mutated TCCYP51, the aromatic ring of Phe\textsuperscript{105} is oriented perpendicular to the heme plane, exposed into the upper portion of the substrate binding cavity (Fig. 4B), whereas the side chain of Val\textsuperscript{107} lies opposite to the active site surface of the B' helix (not shown). Orientation of Phe\textsuperscript{105} in the model is quite similar to that of the corresponding residue (Phe\textsuperscript{9}) in the crystal structure of MT-CYP51 with a non-substrate steroid ligand estriol (1x8v). The estriol C4 atom (which, however, does not carry any methyl groups), lies close (3.76 Å) to Phe\textsuperscript{105}, suggesting that if functional orientation of substrates in the plant CYP51 active site is the same as the orientation of estriol in the MT ortholog, then Phe in this position might affect binding of C4-dimethylated sterols, interfering with their 4β-methyl group (35).

Although the TCCYP51 modeling provides strong rationale only for mutation I105F, the decision to test the role of Thr\textsuperscript{107} was made taking into account low amino acid identity between the MT and TC orthologs (27%) and, particularly, to exclude a possibility of a synergistic effect of these two substitutions since the MT-CYP51 (Phe/Thr\textit{versus} Phe/Val in plants and TB (Fig. 4A)) binds and metabolizes all five sterol substrates. Substitution T107V, however, did not cause any changes either as a single mutation or in the double mutant (I105F/T107V), whereas mutation I105F dramatically alters substrate preferences of TCCYP51.

The addition of N or O at a 1:1 molar ratio enzyme to substrate increases the high spin content of I105F to 52 and 39%, respectively, whereas the percentage of substrate-bound molecules in the wild type TCCYP51 under the same conditions is less than 5% in both cases (Fig. 5A). Based on the calculated ratios hs\textsubscript{m}/K\textsubscript{d}, the efficiency of the interaction with N and O in the mutant increases 113- and 32-fold (Table 2). Binding of C4-dimethylated sterols upon substitution of Ile\textsuperscript{105} to Phe, however, remains essentially unaffected. Spectral responses of the mutant to the addition of equimolar amounts of M, D, or L correspond to 31, 24, and 13% low to high spin transition and are similar to that of the wild type protein. Thus, it is likely that in TCCYP51 (e.g. as a result of some differences in the active site topology or orientation of the substrate in comparison to the orientation of estriol in MT-CYP51) Phe at position 105 does not interfere greatly with the β-methyl group of the
C4-doublemethylated substrates upon their binding. Instead, it probably forms additional interactions with the substrates containing the single equatorial C4-methyl group, which Ile cannot. It is not clear why the greatest increase in affinity is observed with N (more than a 2-order-of-magnitude decrease in the calculated apparent \(K_d\)). We believe that it might be connected with the small volume of the N molecule (Fig. 1B), which somehow makes the requirement for the interaction with the aromatic ring of Phe more strict. In this connection it cannot be excluded that the altered spatial position of the bulkier arm of branched Leu provides higher ability for mammalian and fungal CYP51 to interact with C4-monomethyl sterols, although such a mutation (I105L) in TCCYP51 has not been studied.

Enhanced binding of C4-monomethylated substrates corresponds to the profound increase in their rate of catalysis (Fig. 5B, Table 2). All N and more than 95% of O is 14\(^{\text{\text{a}}}/\text{H}\)251-demethylated by I105F within the time when the wild type TCCYP51 produces only a trace of metabolites from these substrates. The turnover number of N conversion increases \(~150\)-fold in comparison to the wild type, and O is metabolized 60-fold faster. As a result, the activity of the mutant with N (4.7 min\(^{-1}\)) is about 2-fold higher than the activity of the wild type enzyme with the preferred C4-dimethylated substrate (M, 2.4 min\(^{-1}\)). Although I105F does not reveal significant alterations from the wild type in spectral response to L, D, and M (Fig. 5A), the rate of their 14\(\alpha\)-demethylation decreases \(~3.5\)-fold regardless of the side chain composition (Table 2), suggesting that the side chain of the sterols is not involved in the interaction with the residue at position 105. This implies that substitution of Ile\(^{\text{\text{a}}}/\text{H}\)105 to Phe in TCCYP51 without influencing the affinity of binding somehow impairs functional orientation/reorientation of C4-dimethylated sterols in the active site of the enzyme during the three steps of catalysis.

**Effect of I105F on Fluconazole Binding**—We tested the influence of the I105F mutation on the interaction of TCCYP51 with fluconazole to investigate whether this phyla-specific residue could also contribute to the large difference in affinity of this chemical antifungal drug for HU (40 \(\mu\)M) and TBCYP51 (0.45 \(\mu\)M) (20), especially taking into account that in the structure of fluconazole-bound MTCYP51 (1ea1) Phe\(^{\text{\text{a}}}/\text{H}\)78, corresponding to Ile105 in TC, Phe105 in TB, and Leu\(^{\text{\text{a}}}/\text{H}\)31 in HU CYP51 (Fig. 4A) contacts the inhibitor. Unlike the HU ortholog, wild type TCCYP51 has an affinity to fluconazole similar to TBCYP51 (\(K_d\) 0.31 \(\mu\)M), and the value determined for the I105F mutant was found to be only a bit lower (\(K_d\) = 0.17 \(\mu\)M) (type II spectral responses upon titration with fluconazole are shown in Supplemental Fig. 3). Thus, mutation I105F, which alters substrate preferences of TCCYP51, has little influence on its interaction with fluconazole.

**Inhibition of TCCYP51 with Substrate Analogs**

High affinity of sterol 14\(\alpha\)-demethylases toward azole derivatives is well known (36) and has broad practical application; imidazoles and triazoles are the most widely used clinical antifungal drugs (10). Relatively strong binding of TCCYP51 to fluconazole makes testing of a larger panel of azole derivatives as potential anti-Chagastic drugs very promising, especially taking into consideration the inhibitory effect of different azoles on TC in vivo (15–17). However, antifungal azoles are known to inhibit other human P450s (38), cause resistance upon long term treatment, and generally have rather limited life time in aqueous solution.

**Alignment**—Alignment was performed by ClustalW1.81 and prepared in ESPript 2.1 programs. Full sequence alignment of the seven trypanosomal CYP51 is shown in Supplemental Fig. 1. B, location of the mutated residue in the molecular model of TCCYP51. In the overall upper view, Phe\(^{\text{\text{a}}}/\text{H}\)105 (red sphere), heme (green surface), and fluconazole (violet sticks) in the active site are shown. Molecular graphics was generated using UCSF Chimera (www.cgl.ucsf.edu/chimera).
solution (17, 39). Several attempts to use CYP51 substrate analogs to inhibit cholesterol biosynthesis in humans have described that 7-oxo, 15-keto, 15-oxime, 15-hydroxy, 26-oxo derivatives of lanosterol are effective as hypcholesterolemic agents (40–43). Derivatives of the 14α-carboxylic acid intermediate of L were shown to have a dual effect in vitro acting as competitive CYP51 inhibitors and as suppressors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (42, 44). Here we have examined binding and inhibition of TCCYP51 with two substrate analogs, 14α-aminomethyl derivatives of L, AL7 and AL8. These compounds have been found effective against Candida infections, although a direct inhibitory effect on CACYP51 has not been tested (45).

In TCCYP51 both 14α-aminomethyl sterols cause type II spectral response with apparent $K_d$ values of 1.3 and 5.1 μM for AL7 and AL8, respectively (Fig. 6). The 4-fold difference in the affinity might imply that Δ7–8 double bond location somehow is more favorable for coordination of the amino nitrogen to the heme iron. However, when M is added after the titration, it competes with the amino derivatives for the enzyme active site, causing practically equal reverse spectral changes in both cases. This suggests that, affinity of the nitrogen coordination calculated from the type II spectral response does not fully reflect total affinity of the enzyme/ligand interaction. Accordingly, the inhibitory effect of both compounds on TCCYP51 catalysis is practically the same. A 2-fold decrease in the rate of 14α-demethylation of M is achieved at an 11- and 14-fold molar excess of AL7 and AL8 over the enzyme, respectively (molar excess of M in the reaction is 25). Thus, the tested amino derivatives of L inhibit the activity of TCCYP51, although the inhibitory effect is 3–4-fold weaker than the inhibition with the clinical antifungal fluconazole, whereas in cultured cells of the parasite the effects are comparable (46).

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It is not excluded that in the bloodstream, forms of TB, which multiply at the conditions of greater access of host nutrients (6) including a relatively high concentration of host L (49), the CYP51 function of switching sterol biosynthesis from endogenous to exogenous precursors to maintain structural sterol homeostasis is more pronounced. Multiplication of TC in the human body occurs intracellularly, so the source of host L for exponentially dividing amastigotes is rather limited. At this stage of the life cycle the sterol biosynthetic pathway in the parasite is simplified, lacking Δ5 and Δ22 desaturase activity (50). As a result, multiplying TC amastigotes do not form ergosterol, the major sterol of the epimastigote and trypamastigote stages (15). However, all the predicted final products in amastigotes are

**FIGURE 5. Influence of the I105F mutation on the substrate preferences of TCCYP51.** A, low (dashed line) to high (solid line) spin shift in the Soret band of TCCYP1 (1 μM), wild type (WT), and I105F to the addition of sterols at molar ratio enzymesubstrate 1:1. B, HPLC profiles of the radiolabeled metabolites formed by the wild type (lower) and I105F (upper) TCCYP51 in the reconstituted reaction after 10 min of incubation at 37 °C at a P450:sterol molar ratio of 1:25. $S$, substrate; $P$, product, L, 14α-carboxylic acid intermediate. Retention time for N, O, D, and M is 25, 26, 27, 31, and 29 min, respectively.

3 G. I. Lepesheva, M. R. Waterman, W. D. Nes, unpublished results.
4 W. Zhou and W. D. Nes, unpublished results.
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14α-demethylated, and the majority of them are 24-methylated or -ethylated. Depletion of sterols with different antifungals not only harms TC membranes but also affects cytokinesis (13, 51). The latter is in good agreement with the hypothesis that 24-ethylated sterols in *Trypanosomatidae* might be essential functionally, acting as regulators of their cell cycle and development (9). Although the exact mode of action of such bioregulatory sterols has not been determined yet, it is possible that the substrate preference of TCCYP51 toward M might be important to supply the branch of the pathway that leads to their production.

Single amino acid substitution in the B’ helix greatly alters substrate specificity of TCCYP51, converting it into a more plant/TB-like enzyme. Thus, the molecular basis of the wild type TCCYP51 substrate preferences in comparison to those of TBCYP51 is certainly connected with the presence of Ile instead of Phe at position 105. Being relatively small in comparison to Phe, the volume of the Ile side chain is probably insufficient for formation of functional complexes with O or N, suggesting that in vivo the products of the TCCYP51 reaction are C4-dimethylated, although the physiological reason for this difference remains to be clarified. Because the newest sequencing results do not support horizontal gene transfer from Euglena in the evolutionary divergence of *Trypanosomatidae* (protozoan ancestor → *Leishmania* → *Trypanosoma*) (52), the Phe105 has probably come from an older organism (i.e., bacteria), and the F105I variation has played an important role in the development of the TCCYP51 substrate preferences, the functional similarity of TCCYP51 to the animal/fungal orthologs being a result of evolutionary convergence. The fact that the 1105F mutant retains the ability to bind and metabolize C4-dimethylsterols implies that either the topology of the TCCYP51 substrate binding cavity has also been changed during evolution or that TCCYP51 has other residues different from those in the TB ortholog that affect 4β-methyl group binding. A recent finding that CYP51 from *Solanum chacoense*, having O as the preferred substrate, retains the ability to 14α-demethylate L (37) provides additional support for the second option.

The essential role of three conserved residues in the B’ helix in the binding and metabolism of the sterol substrates was established by site-directed mutagenesis of MT and HUCYP51 (Y, F, and G, shaded in black in Fig. 4A) (22). These results coupled to the present study of TCCYP51 indicate that although the motif YX(F/L/I)XXpXFGXXV in the substrate recognition site 1 identifies a P450 as a sterol 14α-demethylase, plant-specific Phe or animal/fungi-like Leu (Ile-105 in TC is shown in bold) can be used to predict substrate preferences of new CYP51 family members. Because the six other CYP51 sequences from *Trypanosomatidae* (Fig. 4A) contain Phe in this position, it is likely that in contrast to TC (subgenus *Schizotrypanum*), in the subgenera *Trypanozoon* (TB *brucei* and TB rhodesiense), *Duttonella* (*Trypanosoma vivax*), and *Nannomonas* (*Trypanosoma congolense*) and in the genus *Leishmania* (*Leishmania major* and *Leishmania infantum*) the post-squalene part of the sterol biosynthetic pathway goes through the 14α-demethylolation of C4-monomethyl precursors.

Although in the reconstituted system *in vitro* TBCYP51 prefers O, our studies on the catalytic properties of 24-sterol methyltransferase from TB (we identified zymosterol as the preferred substrate of the enzyme) strongly suggest that in the cells of this parasite, TBCYP51 might predominantly catalyze 14α-demethylation of N (Supplemental Scheme 1). Taking into account that this C4-monomethyl sterol was also identified *in vivo* in *Leishmania* species upon their treatment with antifungal azoles (25, 26) and recently in TB treated with sterol methyltransferase inhibitors, N may well be the preferred substrate of CYP51 in several *Trypanosomatidae* and, thus, is the fifth physiological substrate of sterol 14α-demethylase.

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REFERENCES

1. El-Sayed, N. M., Myler, P. J., Bartholomeu, D. C., et al. (2005) *Science* 309, 409–415
2. Kreier, T., and Julius, P. (1992) *Parasitic Protozoa*, 2nd Ed., Academic Press, Inc., San Diego, CA
3. Morel, C. M., and Lazdins, J. (2003) *Nat. Rev. Microbiol.* 1, 14–15
4. Coura, J. R., and Castro, S. L. (2002) *Mem. Inst. Oswaldo Cruz.* 97, 3–24
5. Urbina, J. A. (2003) *Curr. Opin. Infect. Dis.* 6, 733–741
6. Berriman, M., Gledin, E., Hertz-Fowler, C., et al. (2005) *Science* 309, 416–422
7. Nes, W. R., and McKean, M. R. (1977) *Biochemistry of Steroids and Other Isoprenoids*, pp. 229–270, University Park Press, Baltimore, MD
8. Schaller, H. (2003) *Prog. Lipid Res.* 42, 163–175
9. Roberts, C. W., McLeod, R., Rice, D. W., Ginger, M., Chance, M. L., and Goad, L. J. (2003) *Mol. Biochem. Parasitol.* 126, 129–142
10. Zhou, W., Lepesheva, M. R., Waterman, and W. D. Nes, submitted for publication.
11. W. D. Nes, unpublished results.
