Folding Requirements Are Different between Sterol 14α-
Demethylase (CYP51) from Mycobacterium tuberculosis and Human
or Fungal Orthologs*

Upon sequence alignment of CYP51 sterol 14α-demethylase from animals, plants, fungi, and bacteria, arginine corresponding to Arg-448 of CYP51 in Mycobacterium tuberculosis (MT) is conserved near the C terminus of all family members. In MTCYP51 Arg-448 forms a salt bridge with Asp-287, connecting β-strand 3–2 with helix J. Deletion of the three C-terminal residues of MTCYP51 has little effect on expression of P450 in Escherichia coli. However, truncation of the fourth amino acid (Arg-448) completely abolishes P450 expression. We have investigated whether Arg-448 has other structural or functional roles in addition to folding and whether its conservation reflects conservation of a common folding pathway in the CYP51 family. Characterization of wild type protein and three mutants, R448K, R448I, and R448A, including examination of catalytic activity, secondary and tertiary structure analysis by circular dichroism and tryptophan fluorescence, and studies of both equilibrium and temporal MTCYP51 unfolding behavior, shows that Arg-448 does not play any role in P450 function or maintenance of the native structure. C-terminal truncation of Candida albicans and human CYP51 orthologs reveals that, despite conservation in sequence, the requirement for arginine at the homologous C-terminal position in folding in E. coli is not conserved. Thus, despite similar spatial folds, functionally related but evolutionarily distinct P450s can follow different folding pathways.

Cytochromes P450 (CYP) form a large superfamily of monooxygenases found in organisms from protists to mammals (1). They catalyze oxidative synthesis and metabolism of various kinds of physiologically important lipopholic compounds, such as sterols, fatty acids, hormones, bio-signaling substances or phytochemicals, as well as detoxify xenobiotics, such as drugs, food additives, and environmental contaminants (1, 2). It has been suggested that all P450s have evolved from a common, ancestral gene by duplication, followed by specific mutations that alter substrate specificity (3). Besides having vastly different substrates, cytochromes P450 differ in their intracellular localization and redox partners (4). As a result, they often exhibit low (sometimes less than 20%) amino acid sequence identity (5, 6). However, comparative analysis of known cytochrome P450 crystal structures (6, 7), hydrophathy and secondary structure prediction algorithms (8), and molecular modeling (9) indicates that all P450s have a very similar spatial fold.

Among more than 1500 known forms of P450, sterol 14α-demethylase (CYP51) represents the only CYP gene occurring in different biological kingdoms with essentially the same metabolic role (10). Via three successive monooxygenation reactions, it catalyzes the removal of the 14α-methyl group from cyclized precursors in sterol biosynthetic pathways (11, 12). In animals CYP51 participates in cholesterol biosynthesis. Fungal and plant isoforms are involved in the synthesis of ergosterol and phytosterols, respectively. Phylogenetic analyses based on protein sequence data have shown that eukaryotic CYP51s are joined into a distinctive evolutionary cluster with bacterial CYP51-like proteins (13). These findings led to a suggestion that CYP51 is one of the most ancient CYP families, which arose in the prokaryotic era before divergence of eukaryotic branches and has been distributed into major biological kingdoms concomitant with their diversification (12).

Identification of sterol 14α-demethylase in M. tuberculosis (14, 15) has provided the opportunity for structural studies of one of the evolutionary oldest CYP gene products. Unlike eukaryotic isoforms, which are localized in the endoplasmic reticulum, MTCYP51 does not contain the N-terminal signal-anchor sequence and is water-soluble. This has made it possible to obtain protein crystals and to determine the MTCYP51 structure (16). In the MTCYP51 crystal structure, Arg-448 (β-strand 3–2) ties together different regions of the molecule by forming a salt bridge with Asp-287 (J-helix) and multiple weak interactions with main chain atoms of amino acid residues 412–414 (junction L-helix-β-strand 3–3). The role of Arg-448 in the MTCYP51 structure/function relationship has been investigated, and effects of C-terminal truncation in bacterial and eukaryotic (Candida albicans and human) isoforms of sterol 14α-demethylase have been compared. It is found that presence of Arg-448 or another amino acid at this position in MT-CYP51 is essential for proper folding but not for maintenance of its structure or for catalytic function. However, in eukaryotic CYP51, the C terminus is not even required for folding. We conclude that, upon heterologous expression in bacterial cells, folding of prokaryotic and eukaryotic forms of CYP51 follows different pathways.

Received for publication, March 29, 2001, and in revised form, May 21, 2001
Published, JBC Papers in Press, May 23, 2001, DOI 10.1074/jbc.M102767200

*This work was supported by Grants GM37942 and ES09267-32 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡Permanent address: Inst. of Bioorganic Chemistry, National Academy of Sciences of Belarus, Minsk, 220141, Belarus. Tel.: 172-637274; E-mail: lepesh@nsi.iboch.ac.by.
§To whom correspondence should be addressed: Dept. of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146. Tel.: 615-322-3318; Fax: 615-322-4349; E-mail: michael.waterman@mcmail.vanderbilt.edu.
†The abbreviations used are: CYP, P450 gene or protein; CYP51, sterol 14α-demethylase cytochrome P450; MTCYP51, sterol 14α-demethylase from M. tuberculosis; HPLC, high performance liquid chromatography; DHL, dihydrolanosterol; bp, base pair(s); PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid.
**MATERIALS AND METHODS**

Restriction endonucleases and other modifying enzymes were purchased from New England Biolabs (Beverly, MA). Reagents for bacterial growth were from Difco (Sparks, MD). 24-[3H]Dihydrolanosterol (DHL) was a generous gift from Dr. J. Trzaskos (DuPont Merck Pharmaceutical Co, Wilmington, DE), and Triton WR 1339 was from Serva (Heidelberg, Germany). Other chemicals were purchased from Sigma.

Recombinant *E. coli* flavodoxin and flavodoxin reductase were expressed and purified as described previously (17).

**Site-directed Mutagenesis**—To insert a four-histidine tag at the N terminus of MTCYP51, the cDNA cloned in pET17b expression vector, Novagen (Madison, WI) (15) was digested at NdeI-XhoI sites (1 to 20 bp) and two annealed oligos, forward (5′-TATGCATCACCATCA-GCCGGGTGCCATTGATGGTGATGCA-3′) and reverse (5′-GGGGTTGATCATCGTTTGTAACGGATACTGG-3′), were ligated between the restriction sites (histidine codons are underlined). The ligation mixture was transformed into *E. coli* BL21 Blue cells (Stratagene, La Jolla, CA) and resultant colonies analyzed by PCR using forward T7 promoter primer, and reverse **5′-CCGGGGTAGTGCAACAGCGCTGTGATGGTGATGCA-3′**) and reverse (5′-CCGGGGTAGTGCAACAGCGCTGTGATGGTGATGCA-3′)) and reverse (5′-CCGGGGTAGTGCAACAGCGCTGTGATGGTGATGCA-3′). Selected colonies were sequenced, and plasmid DNA containing four histidine codons following the initiator methionine was destroyed by silent mutations. The downstream primer **5′-GATGAAACATACAAGTTTCTCATTTTTCCCAAATGATTTCTGC-3′** primer, which is complementary to N-His-tagged MTCYP51 sequence, was used as a template for site-directed mutagenesis of MTCYP51.

As a template for mutagenesis of human CYP51, we used its previously described pCW expression vector (18). C. *albicans* cDNA was amplified by PCR. The forward and reverse primers used to introduce mutations are listed in Table I. Mutations were confirmed by DNA sequencing.

C. *albicans* and human CYP51 were carried out as described previously (18). After expression the cells were pelleted, resuspended (1/10) in 50 mM Tris-HCl (pH 7.5) (containing 1 mM EDTA, 100 mM NaCl, 0.05% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 0.5 mM lysozyme), incubated for 15 min on ice, and then frozen at −70 °C. The total amount of expressed CYP51 was analyzed by size fractionation of *E. coli* proteins in SDS-polyacrylamide gel. To determine the expression level of P450, cells were thawed and sonicated on ice for 6 × 20 s using a Bronson sonifier (Model 250) at duty cycle 30–40 and 50% maximal output. Insoluble material was removed by centrifugation at 100,000 × *g* for 20 min.

**Purification of MTCYP51**—Low expression levels of the MTCYP51 mutants R448K, R448I, R448A, and R448D were modified to modification of previously described (15) purification conditions. The supernatant after sonication was diluted with 50 mM Tris-HCl (pH 7.4), containing 10% glycerol, 0.5 μg/ml lysozyme, and 2 μg/ml ampicillin and applied to a Ni2+-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA) column equilibrated with buffer A containing 20 μM Ni2+ mercaptoethanol and 1 mM imidazole. After extensive three-step washing with buffer A containing 0.1% Triton X-100, buffer A containing 500 mM NaCl and 20 mM Tris-HCl (pH 7.4), containing 10% glycerol (buffer B) and 5 mM imidazole, the P450 was eluted with buffer B containing 50 mM imidazole. The eluate was diluted five times with buffer B and applied onto a Q-Sepharose (Amersham Pharmacia Biotech) column equilibrated with the same buffer. MTCYP51 was eluted with a step gradient of NaCl in buffer B at 0.25 M NaCl. Purified protein was concentrated to 0.2–0.4 mg/ml and dialyzed against 50 mM potassium phosphate (pH 7.4) containing 10% glycerol, and frozen at −70 °C.

**Absorbance Spectra**—Absolute and CO difference absorbance spectra were taken using a Beckman DU 640 spectrophotometer. Spectrophotometric indices of the purified low spin proteins were calculated by dividing the Soret peak absorbance (417 nm) by the absorbance at 280 nm. Spin state of the ferric P450 samples was estimated from the ratio (ΔA393-470/ΔA417-470). The absence of P420 in the purified protein samples was confirmed by CO spectra, which were also used to measure P450 concentrations and kinetics of P450 denaturation of the reduced carbon monoxide complex. Data from different expression experiments were averaged for MTCYP51 and from eight for *C. albicans* and human CYP51.

**Enzymatic Activity**—Sterol 14α-demethylase activity of wild type and mutant forms of MTCYP51 was determined (15) using a radiolabeled 24-[3H]DHL/cold DHL mixture as substrate (50,000 cpm/25 μl in 25 μl of acetone reaction). Radiolabeled DHL was first purified by HPLC. The molar ratio enzyme/substrate in the reaction mixture was 1/25. *E. coli* flavodoxin/flavodoxin reductase system served as MT-CYP51 electron donor partners (molar ratio 18:2:1). The same mixture with no MTCYP51 was used as a negative control. The final reaction volume was 20 μl and contained 20 μM MOPS (pH 7.4), 50 mM KCl, 5 mM MgCl2, and 10% glycerol. The reaction was stopped by the addition of ethyl acetate. Extracted sterols were dissolved in methanol and analyzed by HPLC using a Waters HPLC equipped with Nova-Pak C18 column (3.9 × 150 mm) and β-RAM radioactivity flow detector (INIS Systems Inc, Tampa, FL). Data were analyzed using Millenium software (15).

**CD Spectroscopy**—CD spectra were recorded on a Jasco J-715 spec-
tropolarimeter (Japan) at 20 °C in thermostated cuvettes with 1-mm optical pathlength for the far UV region and 10-mm pathlength for the visible region. Measurements were conducted in 50 mM potassium phosphate (pH 7.4) containing 1 and 10 mM MTCYP51 for the far ultraviolet and visible regions, respectively. Buffer with no protein was routinely recorded and subtracted from the original spectra. Data from six scans were averaged. To compare pH-induced denaturation of mutants, 2–5 μl of protein was added to 1 ml of 50 mM phosphate buffer at a set pH, giving a final concentration of 1 mM. For guanidine hydrochloride-induced denaturation, the protein aliquot was added as above to 1 ml of 50 mM potassium phosphate (pH 7.4), containing different concentrations of guanidine hydrochloride, and incubated for 20 min at 20 °C prior to CD measurements at 223 nm.

**RESULTS**

**Effect of R448 Mutations on the Level of MTCYP51 Expression**

Absence of spectrally detectable MTCYP51 upon deletion of the four C-terminal residues (–RTVG) was originally observed for C-His-tagged MTCYP51 (not shown). To exclude any influence of C-terminal histidines on C-terminally mutated proteins, we have changed the position of His tag to the N terminus of MTCYP51. Optimization of expression conditions allowed us to obtain about 550 nmol of wild type MTCYP51/liter of culture (Fig. 1A). Deletion of three C-terminal residues (–TVG) does not significantly change P450 expression level, but truncation of the fourth residue (Arg-448) results in no spectrally detectable MTCYP51. Spectrally detected expression levels of P540 correlate well with the results of SDS-PAGE analysis of E. coli soluble fractions (Fig. 1B). At the same time, the amount of total protein with molecular weight corresponding to MTCYP51 (CYP51 polypeptide chain in inclusion bodies) was not changed, indicating that the truncations neither affect total synthesis nor induce in vivo proteolytic degradation of MTCYP51.

Replacement of Arg-448 with lysine, a positively charged residue with shorter side chain, gives about 36% P450 expression relative to wild type. Removal of the positive charge, R448I, decreases expression to 20%. Substitution R448A further reduces side chain length and results in only 5% expressed P450. Mutant R448D containing a negatively charged aspartate at position 448 is not expressed in the P450 form. According to the MTCYP51 crystal structure (16), Arg-448 forms a salt bridge with Asp-287, which is located at the C terminus of the J helix (Fig. 2A). Replacement of Asp-287 with arginine, D287R, decreases the expression of P450 to 13% (Fig. 1A). Swapping the electrostatic partners in the salt bridge (double mutant D287R/R448D) led to absence of spectrally detectable P450.

**Fig. 1.** Expression of MTCYP51 mutants in E. coli. A, spectrally detectable levels of P450 accumulated in E. coli soluble fractions for MTCYP51 wild type and mutants. Numbers in parentheses show expression level of mutants as percentage of the wild type. B, size fractionation of E. coli proteins on a 12% SDS-polyacrylamide gel. I, soluble fraction; II, cell lysate. Line 1, MTCYP51 as a molecular weight marker; line 10, E. coli cells without MTCYP51 expression vector.

**Fig. 2.** Arg-448 in the three-dimensional structure of MTCYP51. A, the overall structure of MTCYP51 (distal view). Helices J and L are in *aqua* and *magenta*, respectively; strand β3–2 is in *yellow*. Arg-448 is shown in *dark blue*, and Asp-287 in *red*. B, interactions of Arg-448 and residues substituted for Arg-448 with neighboring residues. Arg-448 mutations were modeled and figures generated using SWISS-PDB VIEWER (45). Hydrogen bonds and charge pairs between the interacting atoms are marked by *dashed lines*, and distances are given in Å. The H-bond shown between Asp-287 and R448K is hypothetical. The most preferred side chain rotamers were used to model each mutation indicated in the figure.
In addition to the salt bridge with Asp-287, Arg-448 can also bond to a nearby portion of the MTCYP51 molecule. Van der Waals surfaces of its long aliphatic arm tightly adjoin to the surfaces of main chain atoms of residues 412–414, suggesting hydrophobic interactions between them. Dependence of MTCYP51 expression levels on the side chain length of the residue substituted for Arg-448 is in good agreement with this assumption (Fig. 1, Table II). In addition, the crystal structure shows that Arg-448 nitrogens, guanidinium nitrogen N^1, and amide nitrogen, may form hydrogen bonds with main chain carbonyls of Leu-412 and Glu-414, respectively. Although the hydrogen bond between N^4 of Arg-448 and carbonyl of Leu-412 is lost upon mutagenesis, the hydrogen bond between main chain atoms is present in all the mutants (Fig. 2B).

Thus, Arg-448 in MTCYP51 forms a link between the C terminus, the J helix, and the C-terminal part of the L helix. This link might be important for 1) maintenance of MTCYP51 structural integrity, 2) heme binding, 3) catalytic activity, or 4) folding of the newly synthesized polypeptide chain. To distinguish between these possibilities, wild type protein and three Arg-448 mutants have been purified and characterized.

**Characterization of MTCYP51 Mutants**

**Light Absorbance**—Because of the strong influence of Arg-448 replacement on P450 expression level, we expected differences in the isolation efficiency of the mutants. However, the yields of pure proteins after the two-stage purification procedure were very similar (Table II). Absolute absorbance spectra of the purified MTCYP51 mutants are shown in Fig. 3A. They all are in the ferric low spin form and have spectrophotometric indexes (\(\Delta\lambda_{417}/\Delta\lambda_{280}\)) of approximately 1.6–1.7, suggesting that the mutation does not affect heme insertion. The reduced CO complex of the mutated P450s show a Soret maximum at the identical position to that of the wild type protein with no detectable P420. Kinetics of denaturation of the CO complexes measured to estimate heme pocket stability show very similar rates of P450 denaturation for wild type and all the mutants (Fig. 3B). Thus, Arg-448 substitution does not affect heme retention.

**Catalytic Activities**—It has been found for human CYP17A that mutation in the C terminus (20) completely eliminates P450c17 activities. Similar results were obtained for C-terminal truncated CYP2C2 and 2C14 (21). Enzymatic activities were checked in whole cells without recalculation per the amount of properly folded P450 as has been done in the current work. Based on a CYP17A three-dimensional model, the absence of catalytic activity has been explained by disruption of the structure of \(\beta\)-sheet 3 and mispositioning amino acid residues that form the substrate-binding site (22). If this were true for MTCYP51, and Arg-448 were important for maintenance of the local conformation of the substrate-binding site, substitution of Arg-448 must influence catalytic activity of the enzyme. However, all the mutants catalyze DHL 14c-demethylation at about the same rate and efficiency as the wild type (Fig. 3C). This indicates that Arg-448 is not important for the enzymatic function of MTCYP51.

**Circular Dichroism**—Circular dichroism (CD) spectroscopy is a valuable technique for detecting conformational changes in proteins. Based on changes in CD spectra, it has been shown that C-terminal truncation of staphylococcal nuclease leads to formation of a functionally active intermediate lacking a considerable portion of its helical structure, called a molten globule (23). In adrenodoxin, C-terminal truncation alters the \(\alpha\)-helical content and accelerates denaturation (24). To evaluate the probability of structural alterations induced by Arg-448 mutation, we measured CD spectra of wild type MTCYP51 and its Arg-448 mutants in the far ultraviolet (UV) and visible regions. Fig. 4A shows the CD spectra in the far UV region (197–250 nm). They are typical for P450 (25, 26) and superimposable. The \(\alpha\)-helical content estimated from mean residue ellipticity ([\(\theta\])_222) at 223 nm is close to 50%, which is in a good agreement with 44% determined from the MTCYP51 structure. Thus, Arg-448 mutations do not induce CD-detectable changes in the MTCYP51 secondary structure. CD spectra of the mutants in the visible region are also essentially unchanged (Fig. 4B). Since the CD of P450 in the visible region (negative Cotton effect at 420 nm) reflects proximity of heme to neighboring amino acid residues (27), these data imply that the heme environment is also not influenced by Arg-448 mutation. These results indicate that the mutated proteins lacking salt bridge Asp-287–Arg-448 and hydrophobic interactions with the L helix do not show any alterations in polypeptide backbone structure or heme pocket environment and cannot be classified as functionally active molten globules.

We also used CD in the far UV region to compare the equilibrium unfolding behavior of the mutants. For these purposes molar ellipticity at 223 nm, \([\theta]_{222}\), was monitored as a function of pH or guanidine hydrochloride concentration (Fig. 4, C and D). All the samples revealed similar equilibrium unfolding transitions with 50% loss of their \(\alpha\)-helical content at pH 5.3 or guanidine hydrochloride concentration of 0.65 M. Lack of noticeable changes in equilibrium unfolding parameters for MTCYP51 mutants indicates the same number of structurally important intramolecular interactions in the mutants as in the wild type. Thus, we conclude that Arg-448 does not contribute to maintenance of MTCYP51 structural integrity.

**Fluorescence Emission**—Tryptophan fluorescence is widely used to follow fine changes in protein tertiary structure and stability (23, 28). Position of fluorescence emission maximum (330–350 nm) and quantum yield reflect hydrophobicity of the indole ring environment and proximity to tyrosines, which serve as donor for resonance energy transfer. Loss of protein tertiary structure is usually accompanied by red shift of tryptophan emission maximum. In hemoproteins tryptophan fluorescence also strongly depends on the distance from the porphyrin ring, which is a nonradioactive emission acceptor and quenches it. As a result, intrinsic tryptophan fluorescence is usually rather weak or even completely quenched in native

---

**Table II**

Purification and spectral characteristics of MTCYP51 mutants

| Sample      | Side chain length of the 448th residue | Purification yielda | Spectrophotometric indexb | Spin state       |
|-------------|----------------------------------------|---------------------|---------------------------|------------------|
| Wild type   | 8.15                                   | 36                  | 1.65                      | \(\Delta\lambda_{293–470}/\Delta\lambda_{417–470}\) |
| R448K       | 7.07                                   | 38                  | 1.70                      | 0.42             |
| R448I       | 4.64                                   | 38                  | 1.67                      | 0.40             |
| R448A       | 2.15                                   | 35                  | 1.62                      | 0.42             |

a Percentage of yield of detectable P450 in E. coli soluble fraction after two-step purification procedure.
b Spectrophotometric index 1.6 corresponds to 18 nmol of heme/mg of protein (15).

---

28416

C Terminus Is Essential for Folding of CYP51 from M. tuberculosis

---

Downloaded from [http://www.jbc.org/](http://www.jbc.org/) by guest on July 24, 2018
hemoproteins but increases sharply during their denaturation. It makes the emission intensity a sensitive indicator of hemo-
protein unfolding (29, 30). MTCYP51 contains 4 tryptophans
and 11 tyrosines, so its intrinsic fluorescence is not quenched
completely. Fluorescence spectra of the wild type protein and
Arg-448 mutants were found to be very similar in their shape
and had emission maximum at 331 nm, indicating a compact
spatial structure of the protein with tryptophan residues bur-
ried inside.

The only difference we have detected between the wild type
and Arg-448 mutants was in kinetics of pH-induced denatur-
ation (Fig. 3D). Mutants show faster unfolding than the wild
type. A 2.6-fold difference in half-time of increase of fluores-
cence emission was observed for the wild type protein and the
R448A mutant (Table III). This slight increase of denaturation
rate may result from absence of a portion of intrinsic interac-
tions within the mutant proteins (Fig. 2B). However, the dif-
ference is too small to indicate significant changes in the pro-
tein stability upon expression under these conditions in E. coli.

All these approaches used to characterize MTCYP51 mu-
tants show that mutations of Arg-448 do not affect MTCYP51
catalytic activity, heme binding, or secondary or tertiary struc-
ture and do not dramatically change its stability. It means that
Arg-448 is essential in MTCYP51 for proper folding, but its
specific properties (guanidinium group) are not required for
P450 function or maintenance of the structure. We suppose
that Arg-448 (or possibly a part of the positively charged region
(-RYRRR448-)) together with negatively charged sequence
around Asp-287 (-DEL287E-) and hydrophobic area of main
chain atoms of residues 412–414 creates a folding nucleus (31,
32) crucial for the initial stages of packing the polypeptide
chain as it emerges from a polyribosome. Failure of MTCYP51
to fold properly in E. coli in the absence of any amino acid in
position 448 implies that this water-soluble, evolutionary an-
cestor of eukaryotic forms of CYP51 follows a folding pathway
that favors arginine at this position.

**C-terminal Truncation of C. albicans and Human CYP51**

Alignment of MTCYP51 sequence with those of other CYP51
family members from different biological kingdoms including
mammals, plants, and fungi has shown that the amino acid residue corresponding to MTCYP51 Arg-448 is highly conserved (Fig. 5A). The sequence preceding Arg-448 in MTCYP51 is also quite conserved, usually including several positive charges. In accordance with the alignment (Fig. 5B), Asp-287, electrostatic partner of Arg-448, although not conserved in the CYP51 family, is located within a negatively charged cluster containing highly conserved Glu-285.

Based on the hypothesis that conservation of non-functional amino acid residues between proteins with low sequence identity but similar three-dimensional structure might be related to evolutionary conservation of the folding pathway (33), we truncated two other representatives of the CYP51 family, C. albicans and human isoforms, and expressed them in E. coli. C-terminal truncation, including the conserved arginine, decreases spectrally detectable amounts of P450 (Fig. 6) but does not abolish it as is seen for MTCYP51 (Fig. 1A). Removal of up to 20 C-terminal amino acids from each protein sequence did not change this result. Increase of the expression temperature to 24 °C and 28 °C enhanced spectrally detectable P450 expression levels of human and C. albicans CYP51, both wild type and the mutants. The difference between the levels of wild type and C-terminal truncated proteins slightly decreases. In contrast, C-terminal truncated MTCYP51 expressed no P450 at any temperature examined. Furthermore, growing MTCYP51 Arg-448 mutants at different incubation temperatures did not change the ratio between the expression levels of wild type and mutants (data not shown). Taken together, these results indicate that folding pathways of C. albicans and human CYP51 differ from that of MTCYP51.

**DISCUSSION**

It is well known and widely discussed (34, 35) that many functionally and evolutionarily related proteins having quite different amino acid sequences adopt similar three-dimensional structures. Although Anfinsen’s postulate that “all the information required to determine the native conformation of a protein resides in its polypeptide chain” (31) is now generally accepted, there are different hypotheses about mechanisms of protein folding (36–38) and the question of how the one-dimensional information encoded by primary sequence is translated into a unique spatial fold is still an important challenge in molecular biology. In the present work, we have explored two related problems: 1) whether Arg-448, an amino acid residue crucial for MTCYP51 folding, has any additional functional or structural importance in the protein molecule, and 2) whether, upon expression in E. coli, its conservation throughout evolution reflects conservation of the folding pathway within the CYP51 family.

Complete absence of P450 expression after truncation of four C-terminal amino acids from the MTCYP51 sequence is in good agreement with the hypothesis supporting formation of a folding nucleus as a crucial stage in the process of protein folding (38, 39, 40). Electrostatic interaction between the cationic group of Arg-448 and anionic group of Asp-287 appears to be necessary to tie different regions of the newly synthesized polypeptide chain together in a salt bridge, whereas two additional hydrogen bonds and weak hydrophobic forces link the protein C terminus and helix L. Absence of the 448th amino acid as well as substitution R448D leads to MTCYP51 malformation and removal from the E. coli soluble fraction into inclusion bodies as a result of the nucleus decomposition. A successful swap of electrostatic partners has been carried out in the CYP2B11 charge pair Asp-290–Lys-242 (41). Residues forming this salt bridge are surrounded mostly by non-polar or oppositely charged amino acids: -LQEI- and -RNLI-. In contrast, in MTCYP51 there is a negatively charged cluster

**TABLE III**

| Sample  | t_{1/2} (s) | ΔF_{max} (%) |
|---------|------------|--------------|
| WT      | 195.7 ± 3.2 | 254.0 ± 1.7  |
| R448K   | 119.2 ± 7.2 | 291.0 ± 5.8  |
| R448I   | 87.3 ± 4.0  | 263.1 ± 3.5  |
| R448A   | 76.1 ± 2.2  | 241.5 ± 1.9  |

**FIG. 4. CD spectra of MTCYP51 Arg-448 mutants.** CD spectra in the far UV (A) and visible (B) regions were recorded in 50 mM potassium phosphate (pH 7.4) at protein concentrations 1 and 10 μM, respectively. Mean residue ellipticity, [θ]_{R}, is expressed on the basis of the number of amino acids per molecule of MTCYP51. Molar ellipticity, [θ], is expressed on the basis of their molar concentration. Molar ellipticity at 223 nm under pH-induced (C) or guanidine hydrochloride-induced (D) denaturation of MTCYP51 Arg-448 mutants.
4-amino acid truncated MTCYP51.

The experiments in Fig. 1, P450 was never observed upon expression of although variations of expression level were observed as indicated. In experiments, P450 was always detected with these truncation mutants, level of mutants relative to wild type. In eight different expression C. albicans fraction for expression.

E. coli.

Spectrally detectable levels of P450 accumulated in the soluble material (MTCYP51). The alignment was performed using Clustal W1.81 program.

FIG.5. Amino acid sequence alignment of CYP51 family proteins from different biological kingdoms. A, sequence alignment with the MTCYP51 positively charged C-terminal region. B, sequence alignment with the MTCYP51 negatively charged region in the helix J. Charged residues are marked in black. Arg-448 and Asp-287 are indicated according to MTCYP51 sequence. Lines 1–4 correspond to mammalian enzymes, lines 5–7, plant; lines 8–16, fungi; line 17, bacterial (MTCYP51). The alignment was performed using Clustal W1.81 program.

(-DELDEL-) in the region of Asp-287 and a positively charged cluster (-RYRRR-) in the region of Arg-448. Insertion of the opposite charge might lead to incorrect local electrostatic interactions as the nascent polypeptide chain appears and therefore result in protein misfolding. It remains unclear how some amount of the synthesized protein without the positive charge at position 448 (20% for R448I and 5% for R448A mutants) can be expressed in the P450 form. We speculate that other positively charged residues preceding Arg-448 (-RYRRR^448) in the absence of negative charge in their neighborhood may partially compensate for the lack of the positive charge at position 448 in formation of the same nucleation center.

Characterization of purified MTCYP51 mutants has shown that Arg-448 does not play any functional or even structural role in the folding molecule. Slight differences between the wild type protein and its mutants in pH-induced unfolding kinetics are the only indication of some decrease in MTCYP51 stability. The increase of the mutants denaturation rate (Table III) corresponds to mammalian enzymes, positively charged C-terminal region.

The Arg-448 participates in the formation of the same nucleation center.

The increase of the mutants denaturation rate (Table III) correlates well with the number of hydrogen bonds formed by the 448th residue. Meanwhile, the mutants similarity in light absorbance and fluorescence emission parameters, molar ellipticity in both far UV and visible regions, as well as in equilibrium unfolding behavior indicates that the amino acid residue in position 448, which is crucial for proper P450 folding, is not essential for maintenance of the native structure of folded MTCYP51. This can be explained by the hypothesis of so-called short term interactions (42). It has been shown that such interactions take place in the nucleation process of polypeptide chains at the earliest stages of protein folding. They juxtapose different parts of the nascent polypeptide chain and then, after stabilization of the tertiary structure of the molecule by multiple bond networks, lose their crucial importance. In accordance with this hypothesis, Arg-448 in MTCYP51 participates in forming a folding nucleus and ensures efficiency of the proper folding pathway, probably by creating favorable conditions for heme incorporation, but its side chain does not play any important role in the folded molecule.

As the Arg-448 is completely conserved throughout the CYP51 family, evolutionary conservation of the folding pathways in functionally related proteins (33) would mean conservation of the same malfolding effect under C-terminal truncation for other family members. However, our results show that C-terminal truncation of C. albicans and human CYP51 decreases but does not abolish accumulation of properly folded P450 in E. coli. This fact indicates that, even in the most conserved among cytochrome P450 families, phylogenetically younger proteins have acquired ability to follow different folding pathways. Both C. albicans and human CYP51 are membrane proteins bound in the endoplasmic reticulum through a hydrophobic signal anchor sequence at the N terminus, produced very early in the biosynthetic process. This probably causes changes in the composition of nucleation centers, which form the folding nucleus leading to a folding pathway different from that of soluble MTCYP51. Possibly the salt link between homologous regions in eukaryotic CYP51 (Fig. 5) is not even formed. One limitation to interpretation of the results of this study could be that expression in all cases is carried out in the heterologous E. coli system. However, MTCYP51 is soluble just as expected and C. albicans and human forms of CYP51 are associated with the E. coli membrane fraction, just as expected for expression of microsomal P450s in this system. Furthermore, a variety of conditions were changed during E. coli expression, yet the same pattern was always observed. Thus, we believe that the folding pathways in the E. coli heterologous expression system are representative of those present in homologous systems.

Existence of multiple folding pathways has been found for a number of proteins (37, 43, 44), its evolutionary purpose being to help a protein molecule conserve its function in the presence of a large number of point mutations. In the case of CYP51, the altered folding pathways in eukaryotic isoforms apparently
developed to conserve sterol 14a-demethylase function in a protein targeted to a specific membrane location, which is required for association with its electron donor, NADPH cytochrome P450 reductase, shared among a large number of P450 enzymes. Thus, conservation of the non-functional residue (Arg-448), which is essential for folding of MTCYP51, does not mean conservation of the folding pathway in the CYP51 family of proteins.

REFERENCES

1. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Wasmann, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) *Pharmacogenomics* 1, 1–42.
2. Belpaire, F. M. and Bogaert, M. G. (1996) *Acta Clin. Belg.* 51, 254–260.
3. Nelson, D. R., Kamataki, T., Wasmann, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K., and Nebert, D. W. (1993) *DNA Cell Biol.* 12, 1–51.
4. Omura, T., Ishimura, Y., and Fujii-Kuriyama, Y. (1993) *Cytochrome P450*, 2nd Ed., Kodansha, Tokyo.
5. Gotoh, O. (1993) *J. Biol. Chem.* 268, 83–90.
6. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Gunsalus, I. C., and Nebert, D. W. (1996) *Pharmacogenomics* 1, 1–42.
7. Hasemann, C. A., Kurumbail, R. G., Boddupalli, S. S., Peterson, J. A., and Deisenhofer, J. (1995) *Structure* 3, 41–62.
8. Tretiakov, V. E., Degtyarenko, K. N., Uvarov, V. Yu., and Archakov, A. I. (1989) *Arch. Biochem. Biophys.* 275, 429–439.
9. Dai, R., Pincus, M. R., and Friedman, F. K. (2000) *Cell. Mol. Life Sci.* 57, 487–499.
10. Asayama, Y., Noshiro, M., Gotoh, O., Imaoka, S., Fumio, K., Kurosawa, N., Horiiuchi, T., and Yoshida, Y. (1996) *J. Biochem. (Tokyo)* 119, 926–933.
11. Fisher, R. T., Stam, S. H., Johnson, P. R., Ko, S. S., Magolda, R. L., Gaylord, J. L., and Trzaskos, J. M. (1989) *J. Lipid Res.* 30, 1621–1632.
12. Yoshida, Y., Asayama, Y., Noshiro, M., and Gotoh, O. (2000) *Biochem. Biophys. Res. Commun.* 273, 799–804.
13. Yoshida, Y., Noshiro, M., Asayama, Y., Kawanoto, T., Horiiuchi, T., and Gotoh, O. (1997) *J. Biochem. (Tokyo)* 122, 1122–1128.
14. Asayama, Y., Horiiuchi, T., Gotoh, O., Noshiro, M., and Yoshida, Y. (1998) *J. Biochem. (Tokyo)* 124, 684–696.
15. Bellamine, A., Mangla, A. T., Nes, W. D., and Waterman, M. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 8937–8942.
16. Podust, L. M., Poulos, T. L., Waterman, M. R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 3068–3073.
17. Jenkins, C. M., and Waterman, M. R. (1998) *Biochemistry* 37, 6106–6113.
18. Stromstedt, M., Rozman, D., and Waterman, M. R. (1996) *Arch. Biochem. Biophys.* 339, 75–81.
19. Shyadehi, A. Z., Lamb, D. C., Kelly, S. L., Kelly, D. R., Schuon, W. H., Wright, J. N., Corina, D., and Akhtar, M. (1996) *J. Biol. Chem.* 271, 12445–12450.
20. Yanase, T., Waterman, M. R., Zachmann, M., Winters, J. S., Simpson, E. R., and Kang, M. (1996) *Biochem. Biophys. Acta* 1319, 275–279.
21. Chen, C. D., and Kemper, B. (1996) *J. Biol. Chem.* 271, 26677–26681.
22. Auchus, R. J., and Miller, W. L. (1999) *Mol. Endocrinol.* 13, 1169–1182.
23. Gotoh, O. (1992) *Biochem. Biophys. Acta* 1122–1128.
24. Chothia, C., and Lesk, A. M. (1986) *J. Mol. Biol.* 181, 355–365.
25. Anfinsen, C. B. (1973) *Science* 181, 223–230.
26. Schulze, J., Tschop, K., Lehnnerer, M., and Hlavica, P. (2000) *Biochem. Biophys. Res. Commun.* 270, 777–781.
27. Uchida, K., Shimizu, T., Makino, R., Sakaguchi, K., Iizuka, T., Ishimura, Y., Nozawa, T., and Hatano, M. (1983) *J. Biol. Chem.* 258, 2519–2512.
28. van Mierlo, C. P., and Steensma, E. (2000) *J. Biotechnol.* 79, 281–288.
29. Marden, M. C., Hui, Bong Hoo, G., and Stetzkowski-Marden, P. (1986) *Biophys. J.* 49, 619–627.
30. Manyusa, S., Mortuza, G., and Whitford, D. (1999) *Biochemistry* 38, 14352–14362.
31. Plotkin, S. S., and Onuchic, J. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6509–6514.
32. Chothia, C., and Lesk, A. M. (1986) *EMBO J.* 5, 823–828.
33. Chothia, C., and Lesk, A. M. (1986) *EMBO J.* 5, 823–828.
34. Plotkin, S. S., and Onuchic, J. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6509–6514.
35. Plotkin, S. S., and Onuchic, J. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6509–6514.
36. Chothia, C., and Lesk, A. M. (1986) *EMBO J.* 5, 823–828.
37. Plotkin, S. S., and Onuchic, J. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6509–6514.
38. Chothia, C., and Lesk, A. M. (1986) *EMBO J.* 5, 823–828.
39. Plotkin, S. S., and Onuchic, J. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6509–6514.
40. Chothia, C., and Lesk, A. M. (1986) *EMBO J.* 5, 823–828.
41. Plotkin, S. S., and Onuchic, J. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6509–6514.
42. Chothia, C., and Lesk, A. M. (1986) *EMBO J.* 5, 823–828.
43. Plotkin, S. S., and Onuchic, J. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6509–6514.
44. Chothia, C., and Lesk, A. M. (1986) *EMBO J.* 5, 823–828.
45. Plotkin, S. S., and Onuchic, J. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6509–6514.
46. Chothia, C., and Lesk, A. M. (1986) *EMBO J.* 5, 823–828.
47. Plotkin, S. S., and Onuchic, J. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6509–6514.
48. Chothia, C., and Lesk, A. M. (1986) *EMBO J.* 5, 823–828.
49. Plotkin, S. S., and Onuchic, J. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6509–6514.
Folding Requirements Are Different between Sterol 14α-Demethylase (CYP51) from Mycobacterium tuberculosis and Human or Fungal Orthologs
Galina I. Lepesheva, Larissa M. Podust, Aouatef Bellamine and Michael R. Waterman

J. Biol. Chem. 2001, 276:28413-28420.
doi: 10.1074/jbc.M102767200 originally published online May 23, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102767200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 44 references, 11 of which can be accessed free at
http://www.jbc.org/content/276/30/28413.full.html#ref-list-1