TOPOLOGICAL CHANGES IN THE CYP3A4 ACTIVE SITE PROBED WITH PHENYLDAZENE: EFFECT OF INTERACTION WITH NADPH-CYTOCHROME P450 REDUCTASE AND CYTOCHROME B₅ AND OF SITE-DIRECTED MUTAGENESIS

Yoshitaka Yamaguchi, Kishore K. Khan, You Ai He, You Qun He, and James R. Halpert

Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas

(Received June 30, 2003; accepted September 22, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

The active site topology of heterologously expressed CYP3A4 purified from an Escherichia coli expression system was examined using phenyldiazene. Incubation of CYP3A4 with phenyldiazene and subsequent oxidation yielded all four potential N-phenylprotoporphyrin IX regioisomers derived from attack on an available nitrogen atom in pyrrole rings A, B, C, or D (Nₐ:N₍:N₆:N₈ = 6:73:7:13). Further study using 28 active site mutants showed that substitution of residues closer to the heme, Ala-305, Thr-309, or Ala-370, with a larger residue caused the most drastic changes in regioisomer formation, which reflected the location of each amino acid residue in a CYP3A4 homology model. Previous studies have suggested a conformational change in CYP3A4 upon binding of NADPH-cytochrome P450 reductase (CPR) or cytochrome B₅ (b₅). Therefore, regioisomer formation was also compared in the absence of redox partners and in the presence of CPR, b₅, or both. Formation of all four regioisomers in CYP3A4 wild type, particularly the minor ones, was reduced in the presence of b₅. CPR also greatly decreased the three minor isomers but increased the major isomer significantly. The presence of b₅ and CPR restored minor isomer formation and suppressed the enhancement of N₈ formation caused by CPR alone. Interestingly, the effects of the redox partners differed among representative active site mutants. In particular, the increase in N₈ upon substitution of Ala-370 with Phe was significantly reversed in the presence of redox partners, strongly suggesting that a conformational change occurs around pyrrole ring C due to protein-protein interactions between CYP3A4 and CPR or b₅.
that $b_3$ also caused a structural change in CYP3A4, which contributed to stimulation of monooxygenase activities (Yamazaki et al., 1996, 2001). More recently, $b_3$ has been shown to alleviate substrate inhibition of CYP3A4 by triazolam (Schrag and Wienkers, 2001). In addition, a similar structural change in CYP3A4 may occur upon interaction with NADPH-cytochrome P450 reductase (CPR), because basic amino acid residues responsible for interaction of CYP2B4 with $b_3$ or CPR are located on the proximal surface near the heme and mostly overlap (Bridge et al., 1998). A conformational change in P450 caused by CPR binding is also supported by a previous observation that the $K_m$ value of rat CYP2B1 or CYP1A1 was changed by chemical modification of acidic amino acid residues on the surface of CPR that are involved in interaction with the P450 (Strobel et al., 1989).

In the present study, amino acid residues at 14 SRS positions in the CYP3A4 active site were selected and substituted with a smaller or larger side chain. The mutants were purified and tested to validate the use of phenyldiazene as a topological probe. Major changes in $N$-protoporphyrin regioisomer formation were observed in some SRS mutants, which were largely consistent with the location of the residues relative to the heme in the CYP3A4 model. Subsequent comparison of $N$-protoporphyrin regioisomer formation in CYP3A4 wild type and representative SRS mutants in the absence and presence of CPR, $b_3$, or both supplied compelling evidence for a structural change in the CYP3A4 active site upon interaction with redox partners.

**Materials and Methods**

**Materials.** Methyl phenyldiazene carboxylate azo ester was purchased from Research Organics (Cleveland, OH). Imidazole, potassium ferricyanide, diazepam, midazolam, mifepristone, and horse skeleton myoglobin were purchased from Sigma-Aldrich (St. Louis, MO), and 7-benzyloxy-4-(trifluoromethyl)coumarin (7-BFC) was obtained from BD Gentest (Woburn, MA). Recombinant CPR and 7-benzyloxy-4-(trifluoromethyl)coumarin (7-BFC) was obtained from BD Gentest (Woburn, MA). Recombinant CPR and 7-benzyloxy-4-(trifluoromethyl)coumarin (7-BFC) was obtained from BD Gentest (Woburn, MA).

**Expression and Purification of CYP3A4 and Mutants.** CYP3A4 wild type and mutants were expressed as His-tagged proteins in Escherichia coli TOPP3 and purified using Talon metal affinity resin (BD Biosciences Clontech, Palo Alto, CA), as described previously (Domanski and Halpert, 2001; Domanski et al., 2001; Harlow and Halpert, 1998; Khan et al., 2002b,c; He et al., 2003). P450 contents were determined by measuring reduced carbon monoxide difference spectra. Protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) and bovine serum albumin as a standard. The specific contents of CYP3A4 wild type and mutants were 6 to 15 nmol of P450 per mg protein except for L373F (specific content = 3).

**Spectral Binding Studies.** Binding spectra were recorded on a Shimazu-2600 spectrophotometer fitted with a temperature controller. The solution in the sample cuvette contained 1 nmol of CYP3A4 wild type in 1 ml of 100 mM phosphate buffer (pH 7.4). Absolute spectra were measured between 350 and 600 nm using 1 ml of 100 mM phosphate buffer (pH 7.4) as a reference. Then, spectral changes were monitored by adding aliquots of 65 mM methyl phenyldiazene carboxylate azo ester in 1 N KOH up to a final concentration of 0.13 mM to both sample and reference cuvettes.

**Formation and Determination of $N$-Phenylprotoporphyrin IX Regioisomers.** $N$-Phenylprotoporphyrin IX regioisomers were extracted three times with 1 ml of CH$_3$Cl$_2$. The extracts were washed with 1 ml of water and dried under reduced pressure. The dried sample was resuspended in 100 $\mu$l of solvent A for HPLC analysis. The regioisomers were separated using a Partisil ODS-3 column (5 $\mu$m × 250 mm × 4.6 mm; Alltech Associates, Deerfield, IL) by isocratic elution with 65:35 (v/v) solvent A (methanol/0.1 M acetic acid, 6:4:1, v/v) and solvent B (methanol) for 35 min at room temperature. The flow rate was 1.0 ml/min, and regioisomers were monitored at 416 nm. Under these conditions, $N$-protoporphyrin regioisomers $N_B$, $N_A$, $N_C$, and $N_D$ eluted at 18 min, 20 min, 22 min, and 24.5 min, respectively, as confirmed by comparison with the standards formed using horse heart myoglobin (5 nmol).

**Results**

$N$-Phenylprotoporphyrin IX Regioisomer Formation from CYP3A4 Wild Type. The addition of phenyldiazene to CYP3A4 wild type yielded a typical peak at 478 nm with a decrease at 418 nm, as expected for a phenyl-iron complex (Fig. 1). After oxidation using ferricyanide, the products were analyzed by HPLC, revealing four $N$-phenylprotoporphyrin IX regioisomers that were matched with the standard products from myoglobin (Fig. 2). $N_A$ was the main product and represented 73 $\pm$ 2% (mean $\pm$ S.D. of six individual determinations) of total regioisomer formation. $N_B$, $N_C$, and $N_D$ were minor and constituted 6 $\pm$ 2%, 7 $\pm$ 1%, and 13 $\pm$ 2%, respectively, of the total. The amounts of these regioisomers were proportional to the P450 added in a range from 0.5 to 2.5 nmol/0.5 ml (data not shown).

Regioisomer formation was also determined in the presence of MgCl$_2$ (10 mM) and the following CYP3A4 substrates: midazolam (25 or 250 $\mu$M), 7-BFC (100 $\mu$M), mifepristone (100 $\mu$M), and diazepam (250 $\mu$M) (Table 1). There was little effect on any regioisomer formation of MgCl$_2$ or 7-BFC. However, midazolam, mifepristone, and diazepam enhanced total regioisomer formation 1.4- to 1.8-fold with little change in the ratios.

$N$-Phenylprotoporphyrin IX Regioisomer Formation by CYP3A4 SRS Mutants. In previous studies from our laboratory, a
combination of molecular modeling and site-directed mutagenesis was successful in allowing us to identify a number of amino acid residues in the CYP3A4 active site that play a significant role in substrate or effector binding and cooperativity (Harlow and Halpert, 1998; Domanski and Halpert, 2001; Domanski et al., 2001; Khan et al., 2002b,c; He et al., 2003). In this study, 14 SRS residues were selected based on our previous studies and substituted with a smaller or larger amino acid to maximize the effect of the substitution. The formation of each N-phenylprotoporphyrin IX regioisomer by the mutants was always compared with wild type and is presented as a percentage of total regioisomer formation by wild type. In the CYP3A4 molecular model, SRS-1 is in the B/C loop, which is a short distance from the heme and located between the I-helix and H9252-sheet 6-1 (Fig. 3). Three amino acid residues, Phe-108, Ser-119, and Leu-120, were selected in SRS-1. As shown in Fig. 4A, the most drastic change was observed in the Ser-119 mutants. The substitution of Ser-119 with Ala decreased formation of all regioisomers, and Trp substitution increased N_A, N_C, and N_D significantly. In contrast, in the Ile-120 mutants, N_A was enhanced by substitution with Ala and unchanged by Trp. Little change was observed in the Phe-108 mutants.

SRS-2 is in the F-helix, which is at a greater distance from the heme than any other SRS and crosses above the I-helix (Fig. 3). Substitution of Leu-210 with Phe enhanced the formation of N_A, but Ala substitution had little effect (Fig. 4B). As reported previously, Leu-211 and Asp-214, along with Phe-304, play a major role in cooperativity (Harlow and Halpert, 1998; Domanski et al., 2001). Interestingly, a double mutant, L211F/D214E, which loses cooperativity of testosterone hydroxylation, showed reduction of the three minor regioisomers with enhancement of N_A formation.

SRS-4 is in the I-helix, crossing above pyrrole ring B (Fig. 3), and significant changes were observed in some mutants in this SRS, as shown in Fig. 4C. In the CYP3A4 molecular model, Ile-301 is close to Ser-119 in the B/C loop. The Ile-301 Ala and Trp mutants showed changes similar to those in the Ser-119 mutants, consistent with close proximity between these amino acids. Phe-304 is on the opposite side of the I-helix toward the heme and is also involved in cooperativity. Replacement with Trp reduced the formation of the minor regioisomers, as in L211F/D214E. In contrast, the replacement of Phe-304 with Ala enhanced regioisomer formation. Ala-305 and Thr-309 are

### TABLE 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>N_B (Percentage of Control)</th>
<th>N_A</th>
<th>N_C</th>
<th>N_D</th>
<th>Total (Percentage of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7 (10,4)</td>
<td>72 (68,76)</td>
<td>5 (6,5)</td>
<td>16 (17,15)</td>
<td>100 (106,94)</td>
</tr>
<tr>
<td>+ MgCl₂ (10 mM)</td>
<td>7 (5,8)</td>
<td>72 (74,69)</td>
<td>6 (8,5)</td>
<td>15 (13,17)</td>
<td>95 (92,97)</td>
</tr>
<tr>
<td>+ Midazolam (25 μM)</td>
<td>5 (5,6)</td>
<td>74 (72,76)</td>
<td>7 (8,7)</td>
<td>13 (15,12)</td>
<td>136 (128,143)</td>
</tr>
<tr>
<td>+ Midazolam (250 μM)</td>
<td>8 (6,11)</td>
<td>72 (75,70)</td>
<td>8 (8,7)</td>
<td>11 (10,13)</td>
<td>161 (152,169)</td>
</tr>
<tr>
<td>+ 7-BFC (100 μM)</td>
<td>5 (6,5)</td>
<td>72 (67,76)</td>
<td>9 (13,5)</td>
<td>14 (14,14)</td>
<td>103 (100,106)</td>
</tr>
<tr>
<td>+ Mifepristone (100 μM)</td>
<td>10 (9,11)</td>
<td>66 (67,66)</td>
<td>9 (10,8)</td>
<td>15 (14,15)</td>
<td>182 (190,173)</td>
</tr>
<tr>
<td>+ Diazepam (250 μM)</td>
<td>9 (12,7)</td>
<td>63 (60,66)</td>
<td>8 (7,8)</td>
<td>20 (21,18)</td>
<td>140 (144,136)</td>
</tr>
</tbody>
</table>

*All values are the mean of duplicate determinations, which are shown in parentheses.*
located just above the heme, and replacement with a larger amino acid residue caused a significant decrease in all regioisomers. In contrast, the substitution of Thr-309 with Ala caused an increase in $N_A$ formation, whereas the replacement of Ala-305 with Gly showed little effect on any regioisomer.

SRS-5 is in $\beta$-sheets 6-1 and 1-4, which are located just opposite the I-helix (Fig. 3). The most significant changes in regioisomer ratios were observed in these SRS mutants (Fig. 4D). Ile-369 is away from the heme, and replacement with a smaller or larger amino acid residue reduced $N_A$ formation. In contrast, Ala-370 is located above the middle of pyrrole rings A and D in the CYP3A4 molecular model. Replacement with Phe increased $N_C$ and decreased $N_A$. Leu-373 is relatively near pyrrole rings C and D (Fig. 3). Substitution with Phe decreased the three minor regioisomers and increased $N_A$, whereas no significant change was observed in L373A. SRS-6 is in $\beta$-sheets 6-2 and 4-2 below the F-helix and between SRS-4 and SRS-5 (Fig. 3). Leu-479 was the only amino acid residue replaced in SRS-6. The mutants showed the most significant change in $N_B$, with Ala yielding a decrease in $N_B$ and Phe an increase.

**Topological Changes in the CYP3A4 Active Site upon Interaction with CPR and $b_5$.** Previous reports indicated a conformational change in the P450 active site following interaction with CPR or $b_5$ (Strobel et al., 1989; Schenkman and Jansson, 2003). To obtain experimental evidence for such structural changes, N-phenylprotoporphyrin IX regioisomers formed from reaction of phenyldiazene with the CYP3A4 wild type were determined in the absence of redox partners and in the presence of two equivalents of CPR, one equivalent of $b_5$, or both. As shown in Table 2, formation of all regioisomers, particularly the minor ones, was reduced in the presence of $b_5$. A large decrease in the three minor regioisomers was also observed in the presence of CPR, but the main regioisomer was significantly enhanced. Interestingly, $N_C$ and $N_D$ were restored slightly in the presence of both CPR and $b_5$, and the enhancement of $N_A$ formation by CPR alone was suppressed in the presence of CPR and $b_5$. These observations strongly suggested structural changes in the CYP3A4 active site caused by protein-protein interactions between CYP3A4 and CPR or $b_5$.

Structural changes in the CYP3A4 active site upon interaction with redox partners should be influenced by substitution of key amino acid residues. Therefore, a representative mutant in each SRS was selected.

**Fig. 4.** Effects on N-phenylprotoporphyrin IX regioisomer formation of amino acid substitutions in SRS-1 (A), SRS-2 (B), SRS-4 (C), SRS-5 (D), and SRS-6 (E).

The regioisomer profile of SRS mutants was always determined in parallel with wild type (WT). The formation of each regioisomer in SRS mutants is represented as a percentage of the total regioisomer formation by WT. All values are the mean of duplicate determinations.
for study of the effects of redox partners on regioisomer formation (Fig. 5). S119F, which showed an increase in all regioisomers, was selected from SRS-1 (Fig. 5A). As with wild type, all regioisomers were reduced in the presence of \( b_c \). Surprisingly, the greatest decrease, including that of \( N_A \), was observed in the presence of CPR alone. All regioisomers were partially restored in the presence of both proteins, but the total regioisomer formation was only about one-half that under control conditions. In the case of the SRS-2 mutant L211F/D214E, very little of the minor regioisomers was formed under any conditions (Fig. 5B), and the effect of redox partners was less than with the wild type. In the case of T309A, an SRS-4 mutant, no minor regioisomer was detected in the presence of \( b_c \), CPR, or both (Fig. 5C). As shown in Fig. 5D, A370F, an SRS-5 mutant, formed almost equal amounts of \( N_A, N_C \), and \( N_D \). \( N_A \) was strongly reduced in the presence of redox partners, especially CPR, and \( N_A \) was enhanced in the presence of \( b_c \) or both proteins but decreased by CPR alone. In contrast, \( N_C \) formation changed little under any conditions. L479F, an SRS-6 mutant, showed a similar profile of regioisomers to wild type in the presence of redox partners (Fig. 5E). The only difference from the wild type was a slightly increased restoration of minor regioisomers in the presence of both CRP and \( b_c \). Overall, the studies with the mutants confirmed that topological changes occur upon interaction of CYP3A4 with redox partners and suggested regions of the active site that are most sensitive to such changes.

### Discussion

The reaction of phenyl diazene with bacterially expressed CYP3A4 formed one major and three minor regioisomers, and the profile was changed by substitution of SRS residues and interaction with redox partners. Some CYP3A4 substrates, including midazolam, also enhanced total regioisomer formation with little change in the ratio. The fact that midazolam induces a prominent type I spectral change in CYP3A4 (Khan et al., 2002c) excludes spin-state changes as the basis for the altered regioisomer patterns caused by active site substitutions or redox partner binding.

In the CYP3A4 model based on the structures of four bacterial P450 enzymes (Szklarz and Halpert, 1997), SRS-4 and SRS-5 are near the heme (Fig. 3), and the regioisomer formation changes caused by amino acid substitutions in these SRSs were consistent with the location of each amino acid residue in the CYP3A4 active site. As shown in the model, Ala-305 and Thr-309 are the closest amino acid residues to the heme iron, and their replacement with a larger amino acid residue caused the most drastic decrease in all of the regioisomers (Fig. 4C), presumably due to interference with initial phenyl diazene coordination to the heme iron. In addition, Ala-370 is located above the middle of pyrrole rings A and D, and replacement with Phe reduced \( N_A \) but enhanced \( N_C \) formation (Figs. 3 and 4D). In contrast, Leu-373 is a short distance from pyrrole ring C, and substitution with Phe enhanced \( N_A \) with reduction of \( N_C \) (Figs. 3 and 4D). These observations reveal that Phe replacement could interrupt adduct formation on the closest pyrrole ring, while enhancing that on the opposite side.

In contrast, substitution of amino acid residues more distant from the heme also caused large changes in regioisomer formation. Ser-119 (SRS-1) substitution with Ala, Phe, or Trp caused changes in regioisomer profiles similar to the corresponding changes at Ile-301 (SRS-4) (Fig. 4, A and C). These data support the inference from a CYP3A4 homology model that the B’-C loop and I-helix interact at these sites, as suggested for the corresponding residues in CYP2D6 and CYP2C5 (Williams et al., 2000; Kirton et al., 2002). Leu-211, Asp-214, and Phe-304 are known to play a major role in cooperativity, which was lost following the substitution with larger amino acid residues (Harlow and Halpert, 1998; Domanski and Halpert, 2001; Domanski et al., 2001). The altered kinetics of L211F/D214E strongly suggested a structural change in the substrate oxidation site by these substitutions at a more distal effector site. Interestingly, both L211F/D214E and F304W formed decreased amounts of \( N_B, N_C \), and \( N_D \) (Fig. 4, B and C). Because these residues are too distant from pyrrole rings B, C, and D to interrupt phenyl group migration to the respective nitrogen atoms (Fig. 3), the substitutions may change the location of the other SRSs in the active site.

The regioisomer profile was also changed in the presence of redox partners (Table 2), strongly suggesting that protein-protein interactions between CYP3A4 and CPR or \( b_c \) caused a conformational change surrounding the heme. Previous studies revealed that basic amino acids on the proximal surface near the heme were involved in the binding of CYP2B4 and 1A1 with \( b_c \) or CPR (Bridges et al., 1998; Cvrk and Strobel, 2001). Our results indicate that each redox partner affects the binding of the other partner with CYP3A4, which may be due to the overlapping binding sites. Previous work using the apo-protein of \( b_c \) strongly suggested that protein-protein interactions, not enhanced second electron transfer, are responsible for enhanced CYP3A4-catalyzed activities (Yamazaki et al., 1996, 2001). Moreover, a conformational change in the CYP3A4 active site upon interaction with redox partners, as suggested by this study, was supported by the observation that the \( K_m \) value for testosterone 6β-hydroxylation decreased in the presence of the apo-\( b_c \) (Yamazaki et al., 1996).

Topological information on CYP3A4 was also reported using phenyl diazene with a human lymphoblast expression system (Schrag and Wienkers, 2000). In that report, MgCl₂ dramatically changed the regioisomer ratio, unlike our study (Table 1). However, the profiles of Schrag and Wienkers (2000) in the presence of MgCl₂ were very close to our findings in the presence of CPR and \( b_c \) (Table 2), indicating that both CYP3A4 preparations have a similar conformation of the active site under this condition.

Changes in the regioisomer profile upon interaction with redox partners were different among SRS mutants (Fig. 5). Substitution of Ser-119 with Trp significantly enhanced all regioisomers (Fig. 4), and...
The increases were suppressed in the presence of CPR and $b_5$ (Fig. 5A). The drastic change observed in this SRS-1 mutant strongly indicated a large conformational change in the B'-C loop upon redox partner binding, which is supported by a previous report that some basic amino acid residues in the C- and C'-helix are involved in binding between P450 and CPR or $b_5$ (Bridges et al., 1998). Studies of A370F revealed that redox partners caused the most drastic change in adduct formation with pyrrole ring C, a space surrounded by the B'-C loop, the I-helix, and $\beta$-sheets 6-1 and 1-4 in the CYP3A4 model (Figs. 3 and 5D). Another interesting observation in A370F was the very small change in N$_D$ caused by redox partners (Fig. 5D), in contrast to the decrease in N$_D$ in the case of the other mutants and wild type (Table 2; Fig. 5). The difference revealed that the substitution for Ala-370 might impede a conformational change that covers the space above pyrrole ring D upon redox partner binding. In some SRS mutants, effects on regioisomer profile were quite different between CPR and $b_5$. Bridges et al. (1998) reported that two additional basic amino acids outside the C- or C'-helix were involved in the binding of CYP2B4 to CPR but not $b_5$. These residues are located in a conserved region between the meander and L-helix (Bridges et al., 1998). Since this conserved region also include the Cys coordinating the heme and two amino acids forming hydrogen bonds with the propionate side chains of the two pyrrole rings, as previously reported in CYP2C5 crystal structure (Williams et al., 2000), CPR but not $b_5$ binding to CYP3A4 might affect the accessibility of the heme in the active site.

In conclusion, the data obtained using CYP3A4 SRS mutants validated use of the N-phenylprotoporphyrin IX regioisomer profile to assess topological changes in the CYP3A4 active site. Subsequent experiments in the presence of redox partners supplied crucial evidence that conformational changes in the CYP3A4 active site occur upon interaction with CPR and $b_5$. The present study implies that interaction with redox partners can also change the regioselectivity or stereoselectivity of substrate oxidation. In fact, substrate inhibition of CYP3A4 triazolam 1'-hydroxylation but not 4-hydroxylation has been reported, and the response was altered in the presence of $b_5$, leading to a change in regioselectivity (Schrag and Wienkers, 2001). Studies of representative SRS mutants strongly suggested that redox partner binding to CYP3A4 alters the relative location of the B'-C loop toward the heme, which is consistent with the interaction site of CYP2B4 with redox partners previously reported (Bridges et al., 1998). Our results also suggest that the conformation of CYP3A4 is different in the presence of both redox partners than in the presence of either one alone (Table 2; Fig. 5). Thus, stimulation of CYP3A4-
catalyzed activities by $b_5$ might also involve improved electron transfer following a change in orientation of CPR toward CYP3A4.

**References**


Tuck SF, Peterson JA, and Ortiz de Montellano PR (1992) Active site topologies of bacterial cytochromes P450101 (P450cam), P450108 (P450erf) and P450102 (P450BM-3). *Proc Natl Acad Sci USA* 89:5402–5406.


**TOPOLOGICAL CHANGES IN CYP3A4 ACTIVE SITE**
