The Cytochrome P450 1A2 Active Site: Topology and Perturbations Caused by Glutamic Acid-318 and Threonine-319 Mutations†

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Received November 12, 1992; Revised Manuscript Received December 23, 1992

ABSTRACT: Phenyldiazene reacts with rat liver CYP1A2 expressed in *Saccharomyces cerevisiae* to give a phenyl–iron complex that rearranges to a mixture (N_B:N_A:N_C:N_D = 12:54:14:20, subscript indicates pyrrole ring) of *N*-phenyl-PPIX (PPIX = protoporphyrin) regioisomers. The same isomer pattern is obtained in each instance when the purified or microsomal enzyme reacts with phenyldiazene, indicating that the active site topology is not altered by removal of the protein from the membrane. Reaction of the enzyme with biphenylhydrazine gives a similar distribution of *N*-biphenyl-PPIX isomers, but reaction with (2-naphthyl)-hydrazine only gives the N_C and N_D regioisomers and a trace of the N_A isomer of *N*-(2-naphthyl)-PPIX. The mutations E318D, E318A, and E318V cause relatively minor changes in the observed regioisomer ratios. In contrast, the mutations T319A, T319V, and T319S suppress formation of the N_C and N_D isomers of *N*-phenyl-PPIX. The reaction of T319A with biphenylhydrazine yields major amounts of the N_B adduct rather than the small amounts observed with CYP1A2 and the Glu-318 mutants, but does not give the N_C and N_D regioisomers. Other, less dramatic, changes in the isomer ratios are also observed. The results indicate that the active site of CYP1A2 is open above all four quadrants of the heme group including, to some extent, the region above pyrrole ring B. Pyrrole ring B is completely inaccessible in most cytochrome P450 enzymes. Mutations of Glu-318 cause relatively minor changes in the active site topology, as expected if it is on the periphery of the active site, but mutations of Thr-319 open up the region above pyrrole rings A and B while constricting the region above pyrrole rings C and D. The results suggest a large active site for CYP1A2 in which the "I-helix" is displaced away from pyrrole ring A, as it is in cytochrome $P450_{BM-3}$ but not in cytochrome $P450_{cam}$.

CYP1A2[1] is constitutively expressed in liver but is also induced by isosafrole, polycyclic aromatic hydrocarbons, and 2,3,7,8-tetrachlorodibenzodioxin. It is involved in the metabolism of many drugs and has been implicated in the metabolism of carcinogenic compounds such as 2-aminofluorene [for reviews see Guengerich (1987), Gonzalez (1990), and Wrighton and Stevens (1992)]. The rat liver enzyme has been expressed in *Saccharomyces cerevisiae* (Shimizu et al., 1986) as part of a site-specific mutagenesis effort to identify active site amino acids and to elucidate their roles in determining the substrate specificity of the enzyme (Shimizu et al., 1988, 1989, 1991a,b; Furuya et al., 1989a,b; Krainev et al., 1991, 1992; Hiroya et al., 1992; Ishigooka et al., 1992). These studies suggest that Glu-318 and Thr-319 are located in the active site and interact with the substrate. Mutations of these residues alter the spectroscopic binding constants for iron-coordinating ligands (Shimizu et al., 1989, 1991a), the kinetics of binding of carbon monoxide (Shimizu et al., 1991b), the binding of phenyl isocyanide to the reduced enzyme (Krainev et al., 1991), and the degree of coupling between NADPH turnover and substrate oxidation (Hiroya et al., 1992; Ishigooka et al., 1992). One of the most striking findings is that the T319A mutant loses the ability to demethylate benzphetamine but retains the ability to oxidize 7-ethoxycoumarin (Furuya et al., 1989a).

Efforts have been made, on the basis of sequence alignments (Nelson & Strobel, 1988; Gotoh & Fujii-Kuriyama, 1989), to use the crystal structure of cytochrome $P450_{cam}$ to construct models for the active sites of membrane-bound cytochrome P450 enzymes (Laughton et al., 1990; Morris & Richards, 1991; Zvelebil et al., 1991). Alignments of the CYP1A1 sequence with that of cytochrome $P450_{cam}$ suggest that Glu-318 corresponds to Asp-251 in the bacterial enzyme. Asp-251 sits above Thr-252 in the crystal structure of cytochrome $P450_{cam}$ and forms ionic bridges with Arg-186 and Lys-178 that help to define the active site of the enzyme (Poulos & Finzel, 1987). Thr-319, a highly conserved residue in the cytochrome P450 family, corresponds to Thr-252 in cytochrome $P450_{cam}$ (Ishigooka et al., 1992). Thr-252 in cytochrome $P450_{cam}$ is located above pyrrole ring A of the heme group (Figure 1) (Poulos & Finzel, 1987). A hydrogen bond between the hydroxyl of Thr-252 and the carbonyl of Gly-248 causes a distortion of the I-helix in cytochrome $P450_{cam}$ that has been suggested to provide a binding site for the iron-bound dioxygen molecule (Poulos et al., 1985). Support for a role of Thr-252 in oxygen activation is provided by site-specific mutation of Thr-252 to an alanine, which severely uncouples oxygen utilization from metabolic formation (Imai

† This work was supported by National Institutes of Health Grant GM25515 and by Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan and the Asahi Glass Foundation. Support for the spectrophotometry core facility of the Liver Center (R. Ockner, Director) was provided by National Institutes of Health Grant 5 P30 DK26743, and for the University of California, San Francisco, Computer Graphics Facility (R. Langridge, Director) by Grant RR 1081.

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[1] Abbreviations: cytochrome P450 enzymes are named according to the nomenclature of Nebert et al. (1991) except for bacterial cytochromes $P450_{cam}$ and $P450_{BM-3}$, which in the standard nomenclature are respectively CYP101 and CYP102; PPIX, protoporphyrin IX; heme, iron protoporphyrin IX regardless of the iron oxidation or ligation state; biphenylhydrazine, (4-phenylphenyl)hydrazine; N_A, N_B, N_C, and N_D, the pyrrole ring bearing the *N*-aryl group, where the subscript indicates the pyrrole ring; HPLC, high-pressure liquid chromatography.

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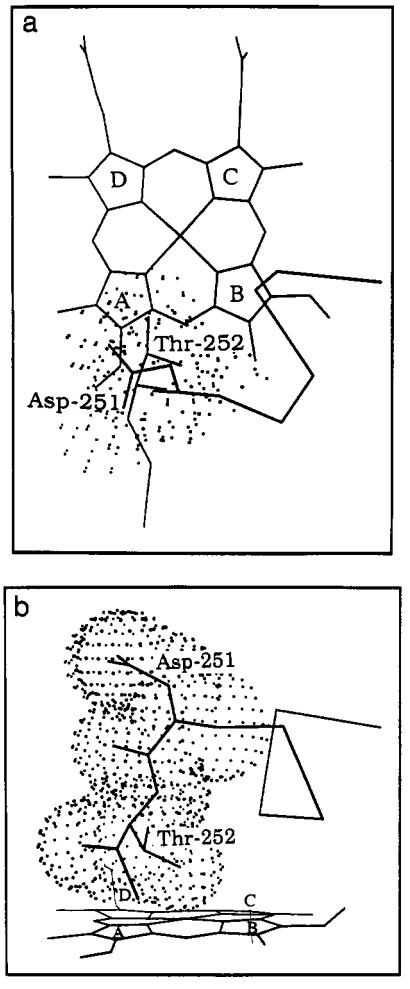

FIGURE 1: Location of Asp-251 and Thr-252 in the cytochrome $P450_{cam}$ active site: (a) top view and (b) side view. Asp-251 and Thr-252 correspond, according to sequence alignments, to Glu-318 and Thr-319 in CYP1A2. The pyrrole rings of the heme are labeled. The speckled surfaces outline the space occupied by the labeled amino acids.

et al., 1989; Martinis et al., 1989). Despite the utility of cytochrome $P450_{cam}$ as a template for the mammalian enzymes, active site models based on the cytochrome $P450_{cam}$ structure must be viewed with skepticism because of the different lengths of the two proteins and the uncertainties in the alignments of their sequences. In the absence of crystal structures, alternative methods must be used to validate inferences concerning the positions of residues in the active sites of the mammalian enzymes.

Recent work has demonstrated that the active site topologies of cytochrome P450 enzymes can be examined with the help of arylhydrazine probes (Swanson et al., 1991, 1992; Tuck & Ortiz de Montellano, 1992; Tuck et al., 1992a,b, 1993). Reaction of the enzymes with arylhydrazines yields relatively stable aryl–iron complexes (Raag et al., 1990), the aryl group of which migrates from the iron to the four nitrogens of the porphyrin within the intact protein when the complex is treated with ferricyanide (Swanson et al., 1991). The regiospecificity of the migration as a function of the size and shape of the aryl group provides direct information on the availability of open space at different heights above each of the four pyrrole rings of the heme group. A topological model referenced to the coordinates of the heme group can be deduced from the data. The approach has been validated by analysis of the active site of cytochrome $P450_{cam}$ and several of its mutants (Tuck et al., 1992b) and has been used to characterize the active site topologies of CYP2B1 and CYP2B2 (Tuck & Ortiz de Montellano, 1992), CYP51 (lanosterol 14α-demethylase) (Tuck et al., 1992a), and other enzymes (Swanson et al., 1991, 1992; Tuck et al., 1992b).

We report here an analysis of the active site topology of CYP1A2 and of the topological alteractions caused by mutation of Glu-318 to an aspartic acid, alanine, or valine, or of Thr-319 to an alanine, valine, or serine. Three probes [phenyldiazene, (2-naphthyl)hydrazine, and biphenylhydrazine] have been used for these topological analyses.

EXPERIMENTAL PROCEDURES

Materials and Methods. Phenyldiazene carboxylate azo ester was purchased from Research Organics, Inc. (Cleveland, OH). Stock solutions of phenyldiazene, typically 2.5 μL of phenyldiazene carboxylate azo ester in 200 μL of argon-saturated 1 M aqueous sodium hydroxide solution, were prepared immediately prior to use and stored on ice. (2-Naphthyl)hydrazine hydrochloride and potassium ferricyanide (99+%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Biphenylhydrazine hydrochloride was synthesized from 4-phenylaniline as previously reported (Tuck & Ortiz de Montellano, 1992). Stock solutions of the hydrazines, typically 25 mM, and potassium ferricyanide (50 mM) were prepared immediately prior to use and were stored on ice. The authenic *N*-aryl-PPIX regioisomers were prepared from arylhydrazine-treated equine myoglobin as previously described (Swanson & Ortiz de Montellano, 1991; Tuck & Ortiz de Montellano, 1992). All reactions were carried out in 100 mM (pH 7.4) potassium phosphate buffer prepared freshly with deionized, doubly glass-distilled water. HPLC was performed throughout with HPLC-grade solvents. All other solvents and reagents were of the highest grade and purity available. Spectroscopic studies were performed on a Hewlett Packard 8452A diode array spectrophotometer equipped with a Lauda RM-6 refrigerating circulator.

Enzymes. Recombinant CYP1A2 and its mutants were prepared as previously described (Shimizu et al., 1991a). Briefly, recombinant enzymes were purified by column chromatography on ω-amino-*n*-hexyl-Sepharose and hydroxylapatite (Bio-Rad, Richmond, CA) after solubilization of yeast microsomes with 0.6% cholic acid. Yeast microsomes and purified enzymes were prepared in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM EDTA, and 1 mM dithiothreitol. Cytochrome P450 concentrations were determined from the difference spectra between the ferrous carbonyl complex of the enzyme and the ferrous, substrate-free form of the enzyme using an extinction coefficient at 450 nm of 91 000 $M^{-} cm^{-1}$. Equine myoglobin (type 1, 95–100%) was purchased from Sigma.

Formation of Iron–Aryl Complexes. For the formation of phenyl–iron complexes of purified cytochrome P450 enzymes, 1 μL of phenyldiazene stock solution was added at 15 °C to a solution of the desired cytochrome P450 (2.5 nmol) in 500 μL of buffer. This resulted in formation of the chromophore of the complex at 478 nm, usually within 10 min, with concomitant loss of Soret absorbance at 416 nm.

For the formation of phenyl–iron complexes of microsomal cytochrome P450 enzymes, 1 μL of phenyldiazene stock solution was added at ambient temperature to a solution of the desired cytochrome P450 (2.5 nmol) in 500 μL of buffer. The mixture was then allowed to sit for 15 min rather than specifically monitoring formation of the phenyl–iron complex because the turbidity of the reaction mixture prevented monitoring complex formation.

Table I: Solvent Mixtures Used To Elute the *N*-Aryl-PPIX Adducts[a]

N-aryl group	% solvent A	% solvent B
phenyl	80	20
biphenyl	60	40
2-naphthyl	70	30

[a] Solvent A is 6:4:1 methanol/water/acetic acid, and solvent B is 10:1 methanol/acetic acid.

For the formation of biphenyl and 2-naphthyl complexes, 20 μL (500 nmol) of arylhydrazine stock solution was added at ambient temperature to a solution of the desired microsomal cytochrome P450 (2.5 nmol) in 500 μL of buffer. After 5 min, 10 μL (1.25 μmol) of potassium ferricyanide stock solution was added to the reaction mixture, followed 20 min later by a further 10 μL (1.25 μmol). A large excess of ferricyanide was required to oxidize an unidentified reducing species in the microsomal suspension.

Active Site-Directed Iron-to-Nitrogen Aryl Shift. To the solution of the purified cytochrome P450 iron–aryl complex was added 2 μL (250 nmol) of potassium ferricyanide stock solution in 0.5-μL aliquots over 1 min. This resulted in immediate loss of the chromophore of the iron–aryl complex. The solution was then added to 5 mL of a freshly prepared solution of 5:95 18 M sulfuric acid/acetonitrile. The mixture was allowed to sit for 1 h at 4 °C before the organic phase was removed in vacuo. The residual aqueous solution was taken up in 2 mL of 0.9 M aqueous sulfuric acid, and the porphyrin adducts were extracted three times with a total of 3 mL of CH_2Cl_2. The organic extracts were concentrated on a rotary evaporator, and the residue was taken up in 150 μL of HPLC solvent A for further analysis (see below). For the complexes of microsomal rather than purified cytochrome P450 enzymes, a single 20-μL aliquot (2.5 μmol) of potassium ferricyanide stock solution was added to the iron–aryl complex and the resulting mixture was allowed to stand for 10 min prior to the same workup.

Substrate-Protection Assays. Phenyldiazene stock solution (1 μL) was added at ambient temperature to microsomal CYP1A2 or its T319A mutant (2.5 nmol) in 500 μL of 100 mM potassium phosphate buffer (pH 7.4). A second equal aliquot of phenyldiazene was added after 10 min. Benzphetamine (500 nmol in 10 μL of buffer) or 7-ethoxycoumarin (325 nmol in 25 μL of methanol) was then added, followed 5 and 10 min later by 1250 and 2500 nmol, respectively, of potassium ferricyanide in 100 mM phosphate buffer. After an additional 5 min, the porphyrin adducts were extracted and prepared for HPLC as described above.

HPLC Analysis of N-Arylprotoporphyrin IX Adducts. A 100-μL sample of the porphyrin dissolved in HPLC solvent A (see below) was analyzed by HPLC on a Hewlett Packard HP 1090 diode array system fitted with an Alltech 5-μm Partisil ODS 3 column. The column was eluted with the solvent mixtures indicated in Table I. Isocratic elution for 30 min was followed by a 1-min gradient to 100% solvent B and 5-min isocratic elution at 100% solvent B. The diode array detector was set at 416 nm with a 4-nm bandwidth. The 416-nm adsorption was corrected for background absorbance by subtracting out the absorbance at 600 nm. *N*-Aryl-PPIX adducts were identified by comparison of their retention times and spectra, and by coelution studies, with authentic *N*-aryl-PPIX isomers. Quantitation of the peak areas was performed using Hewlett Packard integration software. Adduct regioisomers could be detected if they corresponded to 2.5% or more of the total heme in a normal incubation with 2 nmol of enzyme. A zero is indicated in the product ratios if the isomer is not detected and therefore accounts for less than 2.5% of the original heme.

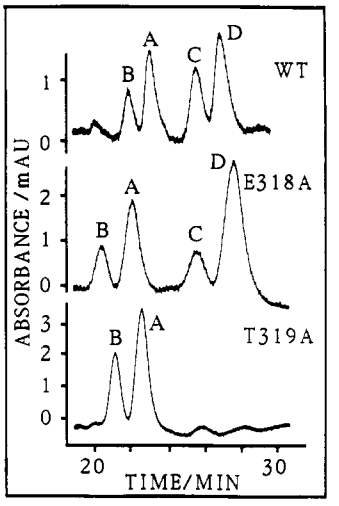

FIGURE 2: HPLC of the *N*-phenyl-PPIX adducts obtained from phenyldiazene-treated microsomal CYP1A2 and its E318A and T319A mutants. HPLC conditions are given under Experimental Procedures. The pyrrole ring bearing the *N*-phenyl group is indicated for each isomer.

RESULTS

Reaction of Phenyldiazene with Purified and Microsomal Recombinant CYP1A2. CYP1A2 and its mutants in which Glu-318 has been converted to an aspartic acid (E318D), alanine (E318A), or valine (E318V), or Thr-319 has been converted to an alanine (T319A), valine (T319V), or serine (T319S), have been expressed in *S. cerevisiae* (Shimizu et al., 1991a). The recombinant enzyme is the only cytochrome P450 present in more than trace quantities in the microsomal preparations from transfected yeast. We have previously shown that shift of the aryl group from the heme iron to the porphyrin nitrogens, the critical step in determination of the active site topology by reaction with arylhydrazines, only occurs within the intact protein if the proximal ligand to the iron is a cysteine thiolate (Swanson & Ortiz de Montellano, 1991; Tuck & Ortiz de Montellano, 1992). The topological analyses can therefore, in principle, be considerably simplified by carrying then out with microsomal, membrane-bound rather than purified enzymes. To establish whether membrane association influences the results, we have determined the ratios of the four *N*-phenyl-PPIX adducts produced by reaction of phenyldiazene with the microsomal (Figure 2) and purified forms of CYP1A2 and six of its site-specific mutants (Table II). The same results, with no more than minor differences that fall within the error of the method (±2–3% for each isomer), are obtained with the purified and microsomal forms of each of the seven enzymes (Table II). These results clearly validate use of the microsomal fractions from transfected *S. cerevisiae* to study the recombinant cytochrome P450 enzymes.

Mutation of Glu-318 to an aspartic acid causes a modest decrease in the extent of pyrrole ring C phenylation, but its mutation to an alanine suppresses detectable reaction with pyrrole ring C and increases that with pyrrole ring B (Figure 2). Replacement of Glu-318 by a valine causes a small increase in D ring arylation at the expense of addition to the A ring. The same changes are observed with the pure and microsomal enzymes. Nevertheless, none of the changes in the *N*-phenyl-PPIX ratios caused by mutating Glu-318 suggest more than subtle alterations in the active site topology. In contrast, Thr-

Table II: N_B:N_A:N_C:N_D Isomer Ratios for the *N*-Aryl-PPIX Products Isolated from Reactions of CYP1A2 and Its Glu-318 and Thr-319 Mutants with Arylhydrazines[b]

	N-phenyl			
enzyme	purified[a]	microsomal[b]	*N*-(2-naphthyl)[b]	*N*-biphenyl[b]
wild-type	10:58:13:19	12:54:14:20	00:05:40:55	12:32:21:34
E318D	13:60:04:23	09:63:05:22	00:13:38:49	06:20:34:40
E318A	21:59:00:20	18:60:00:22	00:00:23:77	11:28:10:51
E318V	13:44:10:32	13:40:13:33	00:00:37:63	13:18:30:39
T319A	40:60:00:00	38:62:00:00	15:31:25:29	34:66:00:00
T319V	18:82:00:00	18:82:00:00	00:26:19:56	08:55:17:20
T319S	35:65:00:00	38:62:00:00	00:16:41:43	13:50:19:18

[a] Experiments carried out with purified recombinant enzymes. [b] Experiments carried out with the microsomal fraction from the yeast expression system. [c] A value of zero ("00") indicates the isomer was not detected. Isomers can be detected if they correspond to 2.5% or more of the original heme in a normal incubation with 2.5 nmol of enzyme.

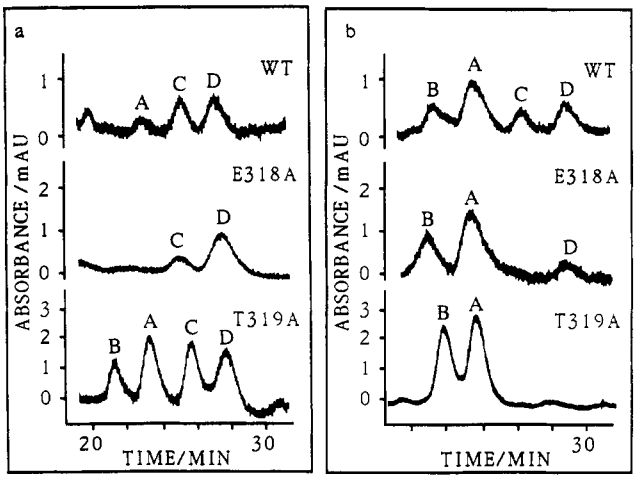

FIGURE 3: HPLC of the *N*-aryl-PPIX adducts obtained from (a) (2-naphthyl)hydrazine-treated or (b) biphenylhydrazine-treated microsomal CYP1A2 and its E318A and T319A mutants. HPLC conditions are given under Experimental Procedures. The letters indicate the pyrrole ring substituted in each of the *N*-aryl-PPIX isomers.

319 mutations give rise to major changes in the *N*-phenyl-PPIX ratios. Thus, mutation of Thr-319 to an alanine or a serine increases reaction with pyrrole ring B while completely suppressing reaction with pyrrole rings C and D (Figure 2, Table II). Mutation of Thr-319 to a valine likewise suppresses addition to pyrrole rings C and D, but increases formation of the N_A rather than N_B isomer. Again, the same changes are seen with the purified and microsomal enzymes, indicating that membrane association does not detectably perturb the active site topology.

Reaction of (2-Naphthyl)hydrazine with Microsomal Recombinant CYP1A2. The reactions of (2-naphthyl)-hydrazine with CYP1A2 and its E318D mutant give roughly comparable amounts of the N_C and N_D isomers, a small amount of the N_A isomer, and none of the N_B isomer of *N*-(2-naphthyl)-PPIX (Table II). In contrast, reaction of (2-naphthyl)-hydrazine with the E318A mutant yields 3 times as much of the N_D as N_C isomer but none of the N_A or N_B isomers (Figure 3). The E318V mutation yields a very similar pattern to that observed with the E318A mutant. Thus, both the alanine and valine mutations suppress N_A formation. Mutation of Thr-319 to an alanine modestly decreases the N_C and N_D isomers of *N*-(2-naphthyl)-PPIX but greatly increases formation of the N_A isomer and, even more remarkably, leads to the appearance in 15% yield of the N_B isomer (Figure 3). The T319V mutation increases N_A at the expense of N_C, and the T319S mutation increases N_A at the expense of N_D. Complete suppression of the N_B regioisomer with the 2-naphthyl probe, the most noticeable difference in the wild-type *N*-aryl regioisomer patterns between the phenyl and naphthyl experiments, is thus reversed by the T319A mutation. Rearrangement of the 2-naphthyl moiety to the nitrogen of pyrrole ring A is also sensitive to these mutations, increasing in the E318D and all the Thr-319 mutants but decreasing in the E318A and E318V mutants.

Reaction of Biphenylhydrazine with Microsomal Recombinant CYP1A2. Reaction of biphenylhydrazine with CYP1A2 gives a pattern of the four *N*-biphenyl-PPIX isomers similar to the *N*-phenyl pattern obtained with phenyldiazene except for a small shift from the N_A to the N_C and N_D isomers (Figure 3, Table II). Mutations of Glu-318 cause some redistribution of the isomers with respect to the wild-type pattern, but no isomer changes by more than approximately 15% and no isomer is suppressed (Table II). The same is true of the T319V and T319S mutations. The T319A mutation, however, suppresses formation of the N_C and N_D isomers while more than doubling the proportions of both the N_B and N_A isomers.

Iron–Nitrogen Shift of the Phenyl Group in the Presence of Substrates. Previous work with CYP2B1 and CYP2B2 has shown that the regioselectivity of the phenyl shift is altered in the presence of substrates of the enzyme, presumably bacause the substrates mask specific regions of the active site (Tuck & Ortiz de Montellano, 1992). An analysis was therefore carried out of the effects of benzphetamine and 7-ethoxycoumarin on the regiochemistry of the phenyl–iron shift in CYP1A2 and its T319A mutant. Benzphetamine and 7-ethoxycoumarin were chosen for this study because their metabolism by the CYP1A2 mutants has already been investigated, and the T319A mutant because the metabolism studies indicate that this mutation causes loss of the benzphetamine but not 7-ethoxycoumarin dealkylation activity (Furuya et al., 1989a; Ishigooka et al., 1992). Nevertheless, the *N*-phenyl-PPIX regioisomer distribution was found to be unchanged when the CYP1A2 phenyl shift was promoted by ferricyanide in the presence of either benzphetamine or 7-ethoxycoumarin. Likewise, the regioisomer pattern was the same for the T319A mutant when the shift was carried out in the presence or absence of either of the two substrates.

DISCUSSION

Cytochrome P450 enzymes expressed heterologously in *S. cerevisiae* are usually present in large excess over the traces of endogenous cytochrome P450 enzymes and therefore can be studied without a significant background of endogenous activity. We have examined the possibility of using the microsomal fraction of yeast rather than the purified enzyme to investigate the active site structure of heterologously expressed cytochrome P450 enzymes because it makes the process less tedious and makes possible the examination of smaller amounts of material. The finding that the ferricyanide-driven in situ iron–aryl shift absolutely requires a thiolate iron ligand circumvents possible problems due to the formation of aryl–iron complexes with other types of hemoproteins (Swanson & Ortiz de Montellano, 1991; Tuck & Ortiz de Montellano, 1992).[2] As shown in Table II, essentially identical values (maximum variance ±2%) are obtained for the percentages of the four *N*-phenyl-PPIX regioisomers generated in the reactions of the purified and microsomal forms of CYP1A2 and six of its mutants with phenyldiazene. These

[2] Unpublished results.

results not only indicate that the analysis can be carried out with the microsomal rather than purified enzymes but also provide direct evidence that the active site topologies of the enzymes investigated are not altered by removal of the proteins from the microsomal membrane. On the basis of these findings, the studies with the (2-naphthyl)hydrazine and biphenylhydrazine probes were only carried out with the microsomal enzymes.

Formation of all four *N*-phenyl-PPIX regioisomers in appreciable amounts in the reaction of CYP1A2 with phenyldiazene (Figure 2, Table II) suggests that the active site of the enzyme is relatively open directly above all four pyrrole rings of the heme. Formation of the N_B regioisomer in 10–12% yield is particularly remarkable because this regioisomer is generally absent. Failure to form the N_B regioisomer in the reaction with cytochrome $P450_{cam}$ is readily explained by the crystal structure of the enzyme, which shows that pyrrole ring B is completely buried beneath the I-helix. Indeed, the contact between Val-247 of the I-helix and pyrrole ring B provides half of the pincer action that is thought to help lock the heme in place in cytochrome $P450_{cam}$. Conservation of the I-helix motif in all other known cytochrome P450 sequences, the structural importance of the contact between the I-helix and pyrrole ring B, and the failure to detect the N_B regioisomer in the reactions of phenyldiazene with several microsomal cytochrome P450 enzymes strongly suggest that pyrrole ring B is similarly masked by the "I-helix" in the active sites of most enzymes, including CYP1A1, CYP2B1, CYP2B2, CYP2B4, CYP2B10, CYP2B11, CYP2E1, CYP3A4, and CYP2C8 (Swanson et al., 1991, 1992; Tuck & Ortiz de Montellano).[2] The only exception to date is cytochrome $P450_{BM-3}$, which gives 13–14% of the N_B regioisomer (Tuck et al., 1992b). Preliminary crystallographic data on cytochrome $P450_{BM-3}$ confirms, in fact, that the "I-helix" is pulled away from pyrrole rings A and B by 1–2 Å, making pyrrole ring B accessible to the phenyl probe.[3] CYP1A2 thus appears to be more closely related to cytochrome $P450_{BM-3}$ than to other cytochrome P450 enzymes with respect to the relative placement of the "I-helix" and the heme group in the active site. This conclusion is strengthened and amplified by the finding that reaction with biphenylhydrazine yields approximately the same percent of the N_B regioisomer (Table II). Migration of the biphenyl moiety, which is much taller than the phenyl group, toward pyrrole ring B suggests that the open space above pyrrole ring B extends to the upper reaches of the active site. Failure of the 2-naphthyl group to migrate to pyrrole ring B suggests, on the other hand, that the volume above pyrrole ring B is tall but not wide because it does not readily accomodate the larger width of the 2-naphthyl group.

Location of Thr-319 near pyrrole ring A of the heme is suggested by the fact that mutations that alter the size of this residue increase the shift of phenyl and biphenyl residues toward pyrrole ring A (Table II). Thr-319 appears to also be near pyrrole ring B because the proportion of the N_B isomer is also increased by mutations of this residue. The side chain of Thr-252, the residue in cytochrome $P450_{cam}$ that aligns with Thr-319 in CYP1A2, sits directly over pyrrole ring A in the crystal structure of both the camphor-bound and phenyl-complexed bacterial protein (Poulos et al., 1987; Raag et al. 1990) (Figure 1). Thr-252 thus prevents migration of aryl groups from the iron to pyrrole ring A in cytochrome $P450_{cam}$ (Tuck et al., 1992b, 1993). The finding that migration toward

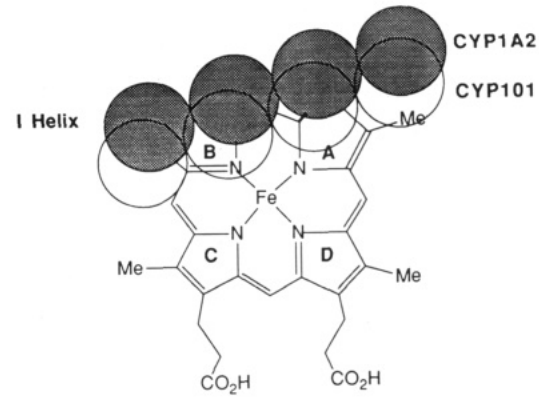

FIGURE 4: Schematic illustration of the proposed difference in the location of the I-helix in cytochrome $P450_{cam}$ (CYP101) and CYP1A2. The I-helix in cytochrome $P450_{cam}$ is represented by the string of open circles and that in CYP1A2 by the string of filled circles. The open circle above pyrrole ring A roughly represents the location of Thr-252 in cytochrome $P450_{cam}$ (see Figure 1). The filled circle above it is the proposed approximate location of Thr-319 in CYP1A2.

pyrrole ring A is not only possible but actually favored by mutations that decrease the size (Ala, Ser) or polarity (Val) of the residue clearly indicates that Thr-319 in CYP1A2 is near, but not directly above, pyrrole ring A. These results are readily rationalized by a displacement of the "I-helix" that opens up the region above pyrrole ring A and, to a lesser extent, pyrrole ring B (Figure 4). The analogy between this lateral displacement and that in cytochrome $P450_{BM-3}$ is strengthened by the finding that phenyl migration to pyrrole ring A dominates in the case of cytochrome $P450_{BM-3}$, as it does with CYP1A2 (Tuck et al., 1992b).

Marked changes in the *N*-aryl-PPIX isomer patterns are observed when Thr-319 is replaced by an alanine, valine, or serine. Shift of the phenyl moiety toward pyrrole rings C and D is suppressed by all three mutations although migration of the biphenyl moiety to give the N_C and N_D isomers is only blocked by the T319A mutation (Table II). A mutation can decrease the proportion of one isomer by increasing the steric congestion above the corresponding pyrrole ring, or by favoring migration to another ring by decreasing the congestion above it. Work with cytochrome $P450_{cam}$ indicates that an increase in the space high above a pyrrole ring can overcome the steric encumbrance provided by a residue directly above the pyrrole ring, presumably by providing the driving force necessary to displace the lower residue (Tuck et al., 1993). In practical terms, this means that the biphenyl and/or 2-naphthyl moieties may shift in directions opposite to those favored by the phenyl group. The present results suggest that the T319V and T319S mutations cause changes in the upper regions of the active site that overcome the steric hindrance detected by the phenyl probe and result in partial migration of the biphenyl moiety to pyrrole rings C and D and the 2-naphthyl moiety to pyrrole ring A (Table II). Furthermore, the fact that Thr-319 mutations suppress migration of the phenyl moiety, and decrease migration of the 2-naphthyl and biphenyl moieties, to pyrrole rings C and D suggests a delocalized conformational change that increases the steric congestion above pyrrole rings C and D while decreasing that over pyrrole rings A and B. Although the decrease in N_C and N_D formation caused by Thr-319 mutations surely reflects a decrease in the barrier to migration toward pyrrole rings A and B, complete suppression of the N_C and N_D isomers suggests that the mutations also have a direct effect in the corresponding, more distant, regions of the active site.

[3] Personal communication from Dr. J. A. Peterson, Southwestern Medical Center, Dallas.

In contrast to the mutations of Thr-319, mutations of Glu-318 to an aspartic acid, alanine, or valine do not cause major detectable alterations in the active site topology. Replacement of this group by an alanine decreases access to pyrrole ring C, but otherwise no clear trend is apparent in the regioisomer patterns obtained with the three probes. Glu-318 thus appears to be less directly involved in determining the active site topology of CYP1A2. Analysis of the location of Asp-251, the corresponding residue in cytochrome $P450_{cam}$, shows that it sits above Thr-252, so that Thr-252 is placed between it and the heme ring (Figure 1). The impact of Asp-251 mutations on the active site topology in the immediate vicinity of the heme group is therefore likely to be dampened by the intervening threonine residue in the bacterial enzyme. The location of Glu-318 relative to Thr-319 and the heme group in CYP1A2 is not known, of course, but the relatively muted and inconsistent impact of Glu-318 mutations on the aryl shift suggests that its relationship to Thr-319 and the heme group may not be unlike that of the corresponding residues in cytochrome $P450_{cam}$.

The failure of benzphetamine or 7-ethoxycoumarin to alter the phenyl shift regiospecificity observed with the parent enzyme or the T319A mutant, given the marked effect of substrates on the regiochemistry of the shift in CYP2B1 and -2B2, can be interpreted in two ways. As there is no independent evidence that the substrates bind to the phenyl–iron complex, it is possible that there is not enough room for the substrates to bind to the phenyl–iron complex. However, the cumulative evidence for a relatively large and open active site supports the alternative that the substrates are actually bound but have sufficient mobility within the active site that they do not selectivity mask one of the heme pyrrole ring nitrogen atoms. It is of some interest, in this context, that addition of dodecanoic or hexadecanoic acids to the cytochrome $P450_{BM-3}$ phenyl–iron complex also does not detectably alter the regiospecificity of the phenyl–iron shift.[2] A precedent therefore exists for the suggestion that substrates need not alter the phenyl shift regiospecificity when it occurs within a relatively large and open active site.

In summary, shift of the aryl group from the iron to all four heme pyrrole nitrogens indicates that the active site of CYP1A2 is relatively open (Table II). The finding that Thr-319 at most partially covers pyrrole ring A, and that pyrrole ring B is accessible to the aryl probes, is best rationalized by outward displacement of the "I-helix" of CYP1A2 with respect to its position in cytochrome $P450_{cam}$ (Figure 4). Thr-252, the corresponding residue in cytochrome $P450_{cam}$, sits directly above, and completely blocks access to, pyrrole ring A. In contrast, preliminary data on cytochrome $P450_{BM-3}$ show that displacement of its "I-helix" by 1–2 Å with respect to the heme coordinates makes pyrrole rings A and B much more accessible than they are in cytochrome $P450_{cam}$.[3] In at least this respect, cytochrome $P450_{BM-3}$ is a better model for CYP1A2 than cytochrome $P450_{cam}$.

ACKNOWLEDGMENT

We are grateful to Yoshinori Murakami for technical assistance in purification of the enzymes.

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