Topological Mapping of the Active Sites of Cytochromes P4502B1 and P4502B2 by in Situ Rearrangement of Aryl–Iron Complexes†

Stephen F. Tuck and Paul R. Ortiz de Montellano*

Department of Pharmaceutical Chemistry, School of Pharmacy, and Liver Center, University of California, San Francisco, California 94143-0446

Received March 13, 1992; Revised Manuscript Received May 6, 1992

ABSTRACT: Cytochrome P4502B1 reacts with phenylhydrazine or phenyldiazene to give an iron–phenyl complex that oxidatively rearranges in situ to the two *N*-phenylprotoporphyrin IX regioisomers with the phenyl group on pyrrole rings A (N_A) and D (N_D) [Swanson, B. A., Dutton, D. R., Lunetta, J. M., Yang, C. S., & Ortiz de Montellano, P. R. (1991) *J. Biol. Chem. 266*, 19258–19264]. The conclusion that the active site of cytochrome P4502B1 is open above pyrrole rings A and D but not B and C is extended here by studies with larger arylhydrazines. The *N*-arylprotoporphyrin IX standards required for product identification were obtained by reaction of the arylhydrazines with equine myoglobin. Cytochrome P4502B1 aryl–iron complex formation followed by oxidative shift of the aryl group produces the following *N*-arylprotoporphyrin IX N_A:N_D regioisomer ratios: phenylhydrazine (39:61), 3,5-dimethylphenylhydrazine (29:71), 4-*tert*-butylhydrazine (25:75), 2-naphthylhydrazine (<2:>98), and 4-(phenyl)phenylhydrazine (87:13). Electron-withdrawing substituents (as in 3,5-dichlorophenyl) prevent the aryl group shift. The increase in the proportion of the N_D regioisomer with increasing bulk of the aryl group suggests that the region over pyrrole ring A is more sterically encumbered than that over pyrrole ring D. The regiospecificity is reversed, however, with 4-(phenyl)phenylhydrazine, which primarily gives the N_A regioisomer. This reversal suggests that the active site has a sloping roof that is higher over pyrrole ring A than pyrrole ring D and that provides a larger steric barrier to the shift of tall aryl moieties than the barrier over pyrrole ring A. An aryl–iron complex is formed with 4-(4-hydrazinophenyl)benzenesulfonic acid, but the aryl ligand appears to be too tall in this instance to shift toward either porphyrin nitrogen. Substrates bind primarily over pyrrole ring D because binding of benzphetamine, aminopyrine, or testosterone to the phenyl–iron complex *before* the oxidative phenyl shift increases the proportion of the N_A regioisomer. Similar results are obtained with cytochrome P4502B2. A refined topological model based on these results is suggested for the active sites of cytochromes P4502B1 and 2B2.

Mammalian cytochrome P450 enzymes are membrane-bound hemoproteins with diverse biosynthetic and metabolic functions (Nelson, 1987; Jefcoate, 1986). Although the primary sequences of more than a hundred of them have been deduced by recombinant DNA methods (Nebert et al., 1991; Black & Coon, 1986; Gonzalez, 1990), the only cytochrome P450 for which a crystal structure is currently available is cytochrome $P450_{cam}$, a soluble enzyme isolated from *Pseudomonas putida* (Poulos et al., 1987). Sequence alignments suggest that several regions are conserved in the mammalian and bacterial enzymes, notably a peptide containing the cysteine to which the heme iron is coordinated and a helix with a threonine thought to be involved in oxygen activation (Poulos et al., 1987; Poulos, 1988). A variety of sequence alignment strategies have been used to fit the mammalian sequences to the cytochrome $P450_{cam}$ template in order to generate models of the tertiary structures of the mammalian enzymes (Nelson & Strobel, 1988; 1989; Edwards et al., 1989; Tretiakov et al., 1989). These models suggest that the structures of the mammalian enzymes are grossly similar to that of cytochrome $P450_{cam}$, except for the presence of a membrane-binding domain at the amino terminus of the mammalian enzymes (Nelson & Strobel, 1989; Tretiakov et al., 1989). The models, however, cannot yet be used with any degree of certainty as predictors of the detailed structures of the mammalian enzymes.

We recently developed a new experimental approach to probe the active-site structure and topology of cytochrome P450 enzymes. This approach is based on the reaction of cytochrome P450 enzymes with phenylhydrazine or phenyldiazene, in the absence of cytochrome P450 reductase or phospholipids, to give stable phenyl–iron complexes with characteristic long-wavelength (476–480-nm) absorption maxima (Jonen et al., 1982; Delaforge et al., 1986; Raag et al., 1990). X-ray crystallography has confirmed that this chromophore is due to a phenyl–iron complex in the reaction of phenyldiazene with cytochrome $P450_{cam}$ (Raag et al., 1990) and has similarly established that the reaction of phenylhydrazine with myoglobin also yields a phenyl–iron complex (Ringe et al., 1984). Extraction of the phenyl–iron complex from myoglobin or cytochrome P450, followed by exposure to acid and an oxidizing agent, results in migration of the phenyl group from the iron to the porphyrin nitrogens to give approximately equal yields of the four *N*-phenyl-PPIX[1] regioisomers (Raag et al., 1990; Swanson & Ortiz de Montellano, 1991; Swanson et al., 1991). The four regioisomers, denoted as N_A, N_B, N_C, and N_D to indicate the pyrrole ring that bears the phenyl group, have been separated and unambiguously identified

† This work was supported by National Institutes of Health Grant GM25515. Support for the core facilities of the Liver Center was provided by Grant 5 P30 DK26743.

* Author to whom correspondence should be addressed.

[1] Abbreviations: PPIX, protoporphyrin IX; heme, iron protoporphyrin IX regardless of the iron oxidation or ligation state; N_A, N_B, N_C, and N_D, the *N*-aryl-PPIX regioisomers with the aryl moiety on the nitrogens of pyrrole rings A, B, C, and D, respectively; HPLC, high-pressure liquid chromatography.

0006-2960/92/0431-6911$03.00/0

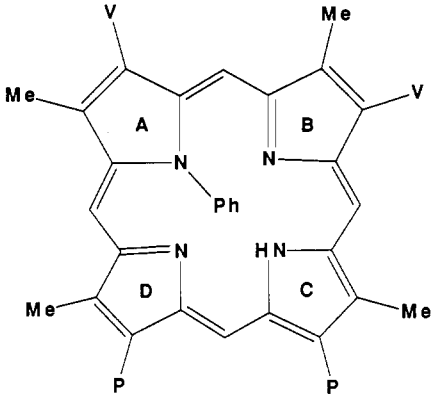


FIGURE 1: Structure of the N_A regioisomer of *N*-phenyl-PPIX with the four pyrrole rings labeled. The N_B, N_C, and N_D regioisomers bear the phenyl group on the nitrogens of pyrrole rings B, C, and D, respectively; V = $-CH{=}CH_2$, p = $-CH_2CH_2CO_2H$.

(Swanson & Ortiz de Montellano, 1991). Ferricyanide oxidation of the undenatured cytochrome P450 (but not myoglobin) phenyl–iron complexes results in in situ migration of the phenyl group to the porphyrin nitrogens (Swanson et al., 1991, 1992; Tuck et al., 1992). However, in contrast to migration of the phenyl group in solution, the steric obstruction provided by active-site residues produces *N*-phenyl-PPIX regioisomer patterns from which the occluded active-site regions can be deduced. Support for the validity of this approach is provided by the finding that it predicts an active-site topology for cytochrome $P450_{cam}$ consistent with that determined by X-ray crystallography (Tuck et al., 1992). Application of this strategy to purified cytochromes P4502B1 and P450B2, isozymes whose sequences differ by only 14 amino acid residues (Suwa et al., 1985), established that their active sites are relatively open above pyrrole rings A and D but closed above pyrrole rings B and C because only the N_A and N_D *N*-phenyl-PPIX regioisomers were formed (see Figure 1) (Swanson et al., 1991). The N_A:N_D ratio for cytochrome P4502B1 is 29:61, whereas that for cytochrome P4502B2 is 63:37, however, suggesting that pyrrole ring D is more open in the former and pyrrole ring A in the latter (Swanson et al., 1991; Tuck et al., 1992). We now report two extensions of the approach that provide more refined topological information and their use to further define the active-site topologies of cytochromes P4502B1 and P4502B2.

EXPERIMENTAL PROCEDURES

Materials. 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, were prepared immediately prior to use and were stored on ice. The hydrochloride salts of 4-*tert*-butylphenylhydrazine, 3,5-dichlorophenylhydrazine, and 2-naphthylhydrazine were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), and were recrystallized from methanol/diethyl ether prior to use. Potassium ferricyanide (99+%) was also from Aldrich. 4-(Phenyl)phenylhydrazine hydrochloride and 3,5-dimethylphenylhydrazine hydrochloride were synthesized from the corresponding amines by a literature procedure (Hunsberger et al., 1956). 4-(4-Hydrazinophenyl)-benzenesulfonic acid, benzphetamine, and aminopyrine were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of the hydrazines, typically 12.5 μmol in 500 μL of buffer, potassium ferricyanide (400 μL, 125 mM), benzphetamine (100 μL, 50 mM), and aminopyrine (100 μL, 250 mM) were prepared immediately prior to use and were stored on ice. The stock solution of testosterone was prepared in methanol (100 μL, 12.5 mM). All buffers were freshly prepared from deionized double-distilled water and were 50 mM MOPS (3-[*N*-morpholino]propanesulfonic acid). HPLC was performed throughout with HPLC-grade solvents. All other solvents and reagents were of the highest grade and purity available.

Enzymes. Cytochromes P4502B1 and P4502B2 were purified from phenobarbital-pretreated rats by the method of Ryan et al. (1982). Cytochrome P450 concentrations were determined from the difference spectra between the ferrous carbon monoxide complex and ferrous substrate-free form of the enzyme using an extinction coefficient of 91 000 M^{-1} cm^{-1} at 450 nm (Omura & Sato, 1964). Equine myoglobin (Type 1, 95–100%) was purchased from Sigma. These spectroscopic assays were carried out on a Hewlett-Packard 8450A diode array spectrophotometer.

Formation of the Iron–Aryl Complexes. To a solution of the desired cytochrome P450 (2.5 nmol in 500 μL) at 25 °C was added 10 μL (250 nmol) of potassium ferricyanide stock solution. Oxidation of the hydrazine with 2 μL (250 nmol) of potassium ferricyanide stock solution resulted in the formation of a new chromophore at 478 nm, usually within 10 min, and concomitant loss of Soret absorbance at 416 nm due to the formation of the aryl–iron σ complex.

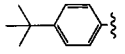
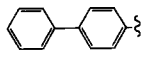
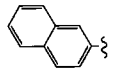
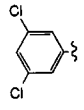
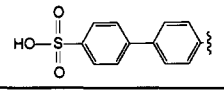
Active-Site-Directed Iron–Nitrogen Aryl Shift. To the solution of the cytochrome P450 iron–aryl complex was added 2 μL (250 nmol) of potassium ferricyanide stock solution. This resulted in immediate loss of the chromophore of the iron–aryl complex. After 5 min, the solution was added to 5 mL of freshly prepared 5:95 18 M sulfuric acid–acetonitrile solution. This was allowed to sit for 1 h at 4 °C before the organic phase was removed in vacuo. The residue was redissolved in 2 mL of 0.9 M aqueous H_2SO_4 and the porphyrin adducts were extracted three times with a total of 3 mL of CH_2Cl_2. The organic extracts were concentrated in vacuo and the residue was dissolved in 150 μL of HPLC solvent A for chromatographic analysis (see below).

HPLC Analysis of N-Aryl PPIX Adducts. A 100-μL aliquot of the sample in HPLC solvent A was analyzed by HPLC on an Alltech Partisil ODS3 5-μm column monitored with a Hewlett-Packard HP 1090 diode array detector. The column was eluted with various mixtures of solvents A and B (Table I): solvent A, 6:4:1 methanol–water–acetic acid; solvent B, 10:1 methanol–acetic acid. Isocratic elution with the indicated solvent mixture for 30 min was followed by a 1-min gradient to 100% B and 5-min isocratic elution at 100% B. The diode array detector, with a 4-nm bandwidth and the reference absorption at 600 nm, was set to monitor the eluent at 416 nm. The *N*-aryl-PPIX adducts were identified by comparison of their retention times and spectra, and coelution, with the myoglobin-derived isomers. Quantitation of the peak areas was performed using Hewlett-Packard integration software.

Preparation of the N-Aryl-PPIX Standards from Myoglobin. To a solution of myoglobin (1 mg, 60 nmol) in 500 μL of buffer was added 10 μL (250 nmol) of the arylhydrazine stock solution. The reaction was allowed to sit at ambient temperature until complete conversion had occurred, as measured by disappearance of the myoglobin Soret band and appearance of the iron–aryl σ-complex chromophore at 430 nm. The porphyrin adducts were extracted and prepared for HPLC as described above.

Effects of Substrates on the Active-Site-Directed Iron–Nitrogen Shift. To a solution of the cytochrome P450 (5 nmol, 500 μL) at 25 °C was added 1 μL of phenyldiazene stock solution. A new chromophore appeared at 478 nm with concomitant loss of Soret absorbance at 416 nm. There

Table I: Solvent Mixtures Used for HPLC of the *N*-Aryl-PPIX Adducts

N-aryl-PPIX adduct	aryl structure	solvent A (%)	solvent B (%)
phenyl		80	20
4-*tert*-butylphenyl		60	40
4-(phenyl)phenyl		60	40
3,5-dimethylphenyl		70	30
2-naphthyl		70	30
3,5-dichlorophenyl		70	30
4-(4-sulfophenyl)phenyl		95	05

appeared to be no further change at either 478 or 416 nm after 10 min. The desired substrate stock solution, either benzphetamine (10 μL, 500 nmol), aminopyrine (10 μL, 1 μmol), or testosterone (10 μL, 250 nmol), was then added, followed 5 min later by 2 μL (250 nmol) of potassium ferricyanide stock solution. This resulted in an immediate loss of the chromophore of the iron–phenyl complex. After a further 5 min, the porphyrin adducts were extracted and prepared for HPLC as already described.

RESULTS

Reaction of Arylhydrazines with Metmyoglobin. In order to obtain standards of the *N*-aryl-PPIX regioisomers, the reactions of metmyoglobin with 3,5-dimethylphenylhydrazine, 4-*tert*-butylphenylhydrazine, 2-naphthylhydrazine, 4-(phenyl)phenylhydrazine, 3,5-dichlorophenylhydrazine, and 4-(4-hydrazinophenyl)benzenesulfonic acid were examined. Reaction of metmyoglobin with each of these arylhydrazines results in the formation of a stable complex with an absorption maximum at ~430 nm analogous to that obtained with phenylhydrazine (Swanson & Ortiz de Montellano, 1991). Aerobic extraction of the aryl–iron complexes into acidic acetonitrile resulted in shift of the aryl group from the iron to the four nitrogens of the porphyrin. The four *N*-aryl-PPIX regioisomers thus formed with each of the arylhydrazines were readily separated by HPLC. In each instance, as observed for the phenyl–iron complex (Swanson & Ortiz de Montellano, 1991), the two faster-eluting regioisomers migrated close to each other and were formed in lower yields than the two slower-eluting regioisomers (Figure 2). The absorption spectra of the first two regioisomers were essentially identical to each other but differed consistently from those of the second set of isomers in that the Soret band was broader for the first two isomers. The bandwidth at half-height averaged approximately 45 nm for the first pair of isomers and 32 nm for the second pair. The same differences in bandwidth are observed for the fully characterized *N*-phenyl-PPIX regioisomers (Swanson & Ortiz de Montellano, 1991). The spectroscopic data indicate that the N_A/N_B pair consistently elutes first

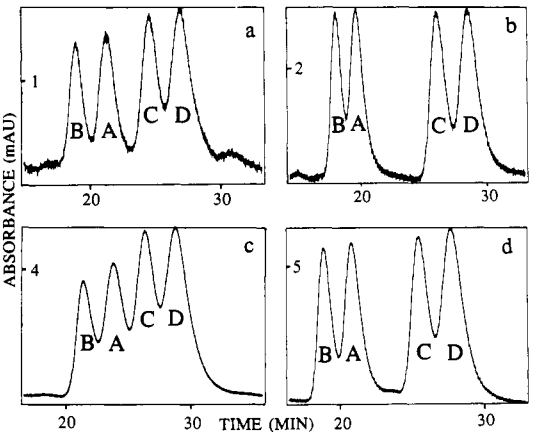


FIGURE 2: HPLC of the *N*-aryl adducts obtained from the reaction of metmyoglobin with (a) 3,5-dimethylphenylhydrazine, (b) 4-*tert*-butylphenylhydrazine, (c) 2-naphthylhydrazine, and (d) 4-(phenyl)-phenylhydrazine. The HPLC conditions are given under Experimental Procedures and in Table I. The proposed N_A, N_B, N_C, and N_D *N*-aryl PPIX regioisomers are labeled.

from the HPLC column. This finding, and the similarities in the elution patterns of the four *N*-aryl-PPIX regioisomers with those of the *N*-phenyl-PPIX isomers, argues that the regioisomer elution order is the same for the *N*-aryl and *N*-phenyl isomers (N_B, N_A, N_C, and N_D, in order of increasing retention time). This inference is strengthened by the fact that the methyl esters of the *N*-methyl- and *N*-ethyl-PPIX regioisomers elute in precisely the same order (Kunze & Ortiz de Montellano, 1981; Ortiz de Montellano et al., 1981). The only exception to this were the adducts obtained with 4-(4-hydrazinophenyl)benzenesulfonic acid, which are quite different due to the sulfonic acid moiety.

Reactions of Arylhydrazines with Cytochrome P4502B1. The reactions of cytochrome P450 enzymes with phenyldiazene produce a stronger spectrum for the phenyl–iron complex than the corresponding reactions with phenylhydrazine (Swanson et al., 1991). Unfortunately, phenyldiazene is the only aryldiazene for which a direct precursor is readily available. To circumvent this difficulty we have generated the diazenes in situ by adding 1 equiv of ferricyanide to an excess of the aryl hydrazines, as done previously by Jonen et al. (1982). An excess of the ferricyanide is to be avoided because it promotes the iron–nitrogen shift. Using this protocol, the reactions of cytochrome P4502B1 with 3,5-dimethylphenylhydrazine, 4-*tert*-butylphenylhydrazine, 2-naphthylhydrazine, 4-(phenyl)-phenylhydrazine, and 4-(4-hydrazinophenyl)benzenesulfonic acid have been found to give stable aryl–iron complexes, as judged by the appearance of an absorption maximum at 478–480 nm. Subsequent incubation of the complexes with excess ferricyanide results in disappearance of the long-wavelength chromophore and recovery of a Soret band with a slightly red-shifted maximum (Figure 3). HPLC analysis of the prosthetic group extracted from the enzyme after reaction with the aryldiazenes and oxidation by ferricyanide shows that only the N_A and N_D regioisomers are formed with 3,5-dimethylphenyldiazene, 4-*tert*-butylphenyldiazene, and 4-(phenyl)phenyldiazene, albeit in different ratios (Figure 4; Table II). Reaction with 2-naphthyldiazene detectably yields only the N_D regioisomer. No *N*-aryl adducts are isolated from the enzyme complex formed with 4-(4-hydrazinophenyl)-benzenesulfonic acid. The regioisomers were assigned by direct chromatographic and spectroscopic comparison with the four regioisomers isolated from the reactions of the arylhydrazines with metmyoglobin.

Alteration of the N-Phenyl-PPIX Regioisomer Pattern by Substrates. The regiochemistry of the oxidative shift of the

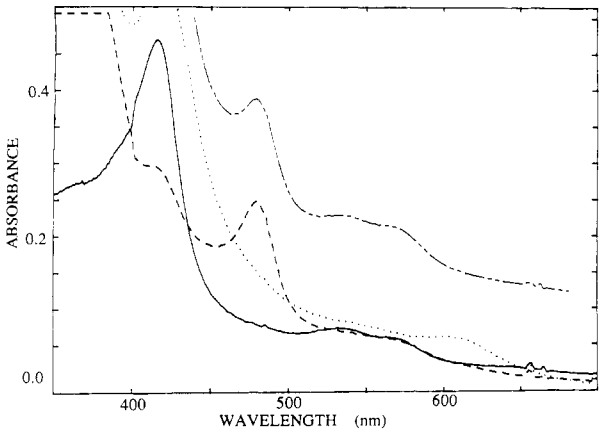


FIGURE 3: Absorption spectra of cytochrome P4502B1 (—), cytochrome P4502B1 after reaction with 2-naphthylhydrazine (– – –) or 4-(phenyl)phenylhydrazine (— -- —) (offset upward for clarity), and after reaction of the 2-naphthylhydrazine-treated enzyme with ferricyanide (···).

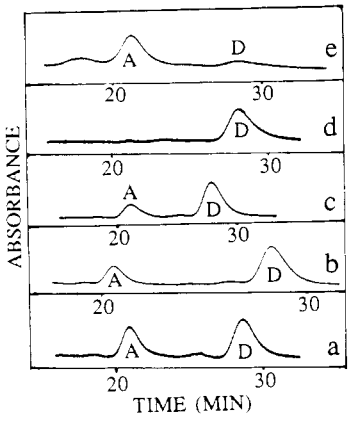


FIGURE 4: HPLC of the *N*-aryl adducts obtained from in situ shift of the complex formed in the reaction of cytochrome P4502B1 with (a) phenyldiazene, (b) 3,5-dimethylphenyldiazene, (c) 4-*tert*-butylphenyldiazene, (d) 2-naphthyldiazene, and (e) 4-(phenyl)phenyldiazene. The HPLC conditions are given under Experimental Procedures and Table I. The N_A and N_D regioisomer peaks are labeled. The approximate *y* scale for each of the plots is 0–4 milliabsorbance units.

Table II: Ratio of the *N*-Aryl-PPIX Regioisomers Formed by Migration of the Aryl Group from the Iron to the Heme Pyrrole Nitrogens within the Intact Protein

	N_A:N_D[a]	
arylhydrazine	P4502B1	P4502B2
phenyl	29:61 (±1)	63:37 (±3)
4-*tert*-butylphenyl	25:75 (±3)	nd[b]
3,5-dimethylphenyl	29:71 (±5)	nd[b]
2-naphthyl	<02:98 (±2)	10:90 (±2)
4-(phenyl)phenyl	87:13 (±2)	86:14 (±1)

[a] The total amount of the N_A + N_D aryl adducts obtained with each of the aryl groups was the same within ±10%. [b] nd = not determined.

cytochrome P4502B1 phenyl–iron complex is altered by benzphetamine (Guengerich et al., 1982), aminopyrine (Guengerich et al., 1982), and testosterone (Guengerich et al., 1982; Wood et al., 1983), three structurally dissimilar substrates of the enzyme. The first two are N-demethylated with turnover numbers of 72 and 85 nmol (nmol of cytochrome P450)$^{-1}$ min^{-1}, respectively, whereas the last one is hydroxylated at the 16 and 17 positions with a 10-fold lower turnover number (Guengerich et al., 1982; Wood et al. 1983; Guengerich, 1987). The phenyl–iron complex of cytochrome P4502B1 was preformed by reaction with phenyldiazene. After the chromophore change indicative of phenyl–iron complex formation was complete (10 min), benzphetamine (1 mM), aminopyrine (2 mM), or testosterone (0.5 mM) was added and the mixture was allowed to equilibrate for 5 min before ferricyanide was added to promote the phenyl shift. HPLC analysis of the *N*-phenyl-PPIX regioisomers thus obtained shows that each of the three substrates increases the proportion of the N_A isomer at the expense of the N_D isomer (Table III), testosterone being the most, and aminopyrine the least, effective in this regard.

Table III: *N*-Phenyl-PPIX Ratios Obtained by Migration of the Phenyl Group from the Iron to the Heme Pyrrole Nitrogens in the Presence of Substrates

	N_A:N_D[a]	
substrate	P4502B1	P4502B2
none	29:61 (±1)	63:37 (±3)
aminopyrine	56:44 (±1)	nd[b]
benzphetamine	48:52 (±1)	74:26 (±5)
testosterone	66:34 (±3)	nd[b]

[a] The total amount of the N_A + N_D aryl adducts that was recovered was the same within ±10%. [b] nd = not determined.

Cytochrome P4502B2. Experiments analogous to those described for cytochrome P4502B1 were carried out with cytochrome P4502B2 with very similar results. Reaction of the enzyme with each of the arylhydrazines resulted in appearance of the chromophore characteristic of the aryl–iron complex (not shown). Subsequent reaction with ferricyanide caused disappearance of the chromophore and formation of the N_A and N_D *N*-aryl-PPIX regioisomers (Table II). The trends in the regioisomer patterns were similar to those obtained with cytochrome P4502B1 in that the 2-naphthyl–iron complex gave rise predominantly to the N_D regioisomer, whereas the 4-(phenyl)phenyl complex gave rise predominantly to the N_A regioisomer (Table II). In accord with the fact that the N_A:N_D ratio for the phenyl–iron complex of cytochrome P4502B2 is biased toward the N_A isomer whereas that for P4502B1 favors the N_D isomer (Table II), the cytochrome P4502B2 2-naphthyl complex predominantly but not exclusively forms the N_D regioisomer, whereas only the N_D isomer is detectably formed from the cytochrome P4502B1 complex. In the presence of benzphetamine, the proportion of the N_A isomer is increased, as it is with cytochrome P4502B1 (Table III). Substrates therefore appear to also bind predominantly over pyrrole ring D in cytochrome P4502B2.

Effect of Electron-Withdrawing Substituents. Reaction of cytochrome P4502B1 with 3,5-dichlorophenylhydrazine is associated with the chromophore shift expected for formation of the 3,5-dichlorophenyl–iron complex (not shown). However, the chromophore is not altered by the addition of excess ferricyanide. Furthermore, when the complex is worked up normally, the four *N*-(3,5-dichlorophenyl)-PPIX regioisomers are obtained in comparable amounts, a pattern indicative of shift of the iron–aryl complex *after* extraction from the protein. The 3,5-dichlorophenyl moiety therefore does not undergo the ferricyanide-mediated in situ iron–nitrogen shift even though the comparably-sized 3,5-dimethylphenyl group does so.

DISCUSSION

The reaction of cytochrome P4502B1 with phenyldiazene, as reported earlier (Swanson et al., 1991), yields a stable phenyl–iron complex. In situ oxidation of the complex results in regioselective migration of the phenyl group to the nitrogens of pyrrole rings A and D of the heme group (Figure 1). This result suggests that the region above pyrrole rings B and C is blocked and is not accessible from the active site. This result is supported by the finding that linear 1-alkynes and

1-alkenes regioselectively alkylate the nitrogens of pyrrole rings A and D, respectively (Kunze et al., 1983). As previously noted, obstruction of the region above pyrrole ring B is consistent with the inference from sequence alignments that a helix equivalent to the I-helix of cytochrome $P450_{cam}$ is widely, possibly universally, conserved (Nelson & Strobel, 1988; 1989; Edwards et al., 1989; Tretiakov et al., 1989) because the I-helix in the bacterial enzyme covers pyrrole ring B and renders its nitrogen inaccessible from the active site (Tuck et al., 1992). The present study provides additional topological information that allows us to further define the earlier active-site model.

Formation of iron–phenyl complexes with all of the aryl-hydrazines employed in this study provides a measure of the height of the roof directly above the heme iron atom in the active site. The most demanding ligands in this regard are the 4-(phenyl)phenyl and 4-(sulfophenyl)phenyl moieties, which can be estimated to rise to a vertical height, respectively, of approximately 10 and 13 Å above the heme plane.[2] All the other ligands are shorter than this and are therefore accommodated by this height. Formation of the aryl–iron complex provides less definite information on the volume of the space above the iron because the ligands, except for the substituents, are composed of planar aromatic rings. The planar ligands could fit into a narrow crevice but a very narrow active site is not compatible with the present data because it would not allow the aryl ligands to migrate to two nitrogens separated by an angle of 90°. It is not possible, however, to deduce from aryl–iron complex formation how much of the cylinder that is defined by rotating the ligands 360° about the iron–carbon bond is actually available for substrate occupancy.

The fact that increasing the bulk of the phenyl group by adding two methyl groups or a *tert*-butyl group decreases the proportion of the N_A regioisomer suggests that the region above pyrrole ring D is less sterically encumbered than that above pyrrole ring A. This is confirmed by the finding that the additional bulk of the 2-naphthyl group results in essentially exclusive migration of the 2-naphthyl ligand to the nitrogen of pyrrole ring D. The observation that more space is available above pyrrole ring D than pyrrole ring A suggests that substrates are primarily bound over the former rather than the latter. Support for this inference is provided by the fact that binding of aminopyrine, benzphetamine, and testosterone to the cytochrome P4502B1 phenyl–iron complex *before* the oxidative shift is initiated increases formation of the N_A regioisomer at the expense of the N_D regioisomer. Binding of the three substrates over pyrrole ring D predicts, as found, increased migration of the phenyl group to the nitrogen of pyrrole ring A. It is notable, furthermore, that the proportion of the N_A regioisomer increases in going from the smallest (aminopyrine) to the largest (testosterone) substrate (Table III).[3]

In contrast to favored formation of the N_A regioisomer with 4-*tert*-butylphenyl and other ligands, the 4-(phenyl)-phenyl group primarily yields the N_D regioisomer. The primary difference between the 4-*tert*-butyl and 4-phenyl substituents is the increased height of the 4-phenyl moiety. This difference in height therefore presumably accounts for the fact that the 4-phenyl substituent causes the ligand to shift preferentially toward the nitrogen of pyrrole ring D, whereas all the other substitutions examined increase the shift of the ligand toward the nitrogen of pyrrole ring A. This dichotomy can be rationalized by postulating that the roof of the active site slopes downward from the region above pyrrole ring A toward the region above pyrrole ring D. For relatively short ligands, the direction of the shift is reponsive to the higher steric encumbrance in the vicinity of pyrrole ring A. However, as the height of the ligand increases a point is reached where it can shift toward pyrrole ring A without bumping into the roof but cannot similarly shift toward pyrrole ring D because of the lower roof in that region. If the interaction with the roof is more severe than that with the residues above ring A, the aryl group will shift toward the nitrogen of pyrrole ring A. Support for a sloping roof with its apex directly above the iron is provided by the formation of a 4-(sulfophenyl)phenyl–iron complex that does not rearrange to *N*-aryl heme products when oxidized by ferricyanide. This suggests that the 4-(sulfophenyl)phenyl ligand is too high to shift toward either nitrogen, although the alternative cannot be excluded that the iron–nitrogen shift is prevented by electrostatic rather than steric interactions of the ionized, negatively charged sulfonic acid group with active-site residues. A schematic representation of the active-site topology predicted by these results is given in Figure 5.

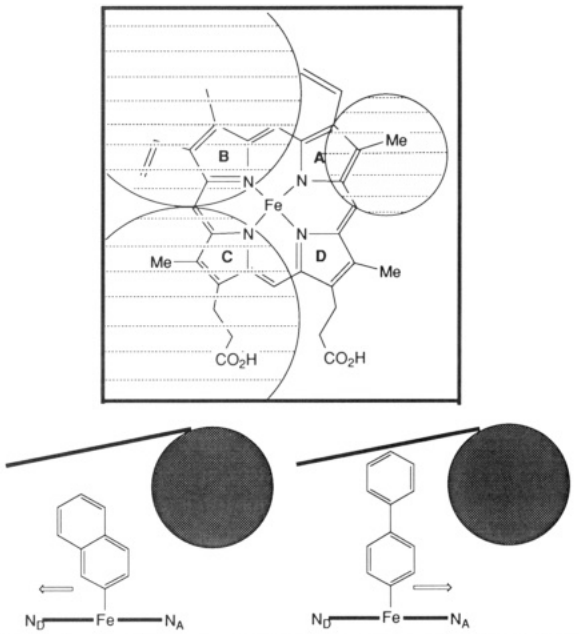


FIGURE 5: Topological model of the active site of cytochrome P4502B1 and 2B2. The data suggest that the steric block over pyrrole ring A is larger (or that over pyrrole ring D smaller) in cytochrome P4502B2 than in cytochrome P4502B1.

The active site of cytochrome P4502B2 is shown by the same aryl shift method to be similar to that of cytochrome P4502B1, except that the region above pyrrole ring D is more congested relative to the region above pyrrole ring A. Thus, the *N*-phenyl-PPIX N_A:N_D ratio obtained from the phenyl–iron complex favors N_A in cytochrome P4502B2 but N_D in cytochrome P4502B1. Nevertheless, the change in the N_A:N_D ratio caused by changes in the aryl group structure (Table II) and by the presence of substrates (Table III) are very similar for both enzymes. The differences in the catalytic activities of the two enzymes therefore reflect a relatively subtle structural perturbation caused by one or more of the

[2] The height of the biphenyl complex is based on a length of 2.8 Å for the phenyl group obtained by triangulation (0.7 + 1.4 + 0.7 Å) using a C–C bond length of 1.4 Å (Stanley & Adams, 1930), a phenyl–phenyl distance of 1.4 Å, and Fe–C bond length of 1.9 Å taken from the X-ray structure of the cytochrome $P450_{cam}$ complex (Raag et al., 1990), and a C–H bond length of 1.0 Å for the uppermost hydrogen (i.e., 1.0 + 2.8 + 1.4 + 2.8 + 1.9 ≃ 10.0 Å). Replacement of the C–H by a sulfonic acid group should increase the height by about 3 Å.

[3] The present argument suggests that the 4-(phenyl)phenyl shift should occur more slowly than the other shifts but it has not been possible to confirm this because the shifts occur too rapidly to be followed by static spectroscopic methods.

14 amino acid differences (Suwa et al., 1985), possibly the relative difference in the steric hindrance over pyrrole rings A and D detected in these studies, rather than a gross difference in their active-site structures. The active-site model in Figure 5 applies to cytochrome P4502B2 as well as cytochrome P4502B1 if it is understood that a small difference exists in the size or positioning of the steric barrier(s) above ring A in the two enzymes. One possible source of the difference in the two active sites is the single amino acid difference in their I-helix sequences (Thr_{2B1}-303 versus Ala_{2B2}-303) because the I-helix sits on the heme group (Poulos et al., 1987; Poulos, 1988; Suwa et al., 1985), but it is not possible with the available information to determine the role, if any, of this amino acid replacement.

Model studies with iron porphyrins have shown that one-electron oxidation of the phenyl–iron (Fe^{III}–Ph) complexes to the corresponding Fe^{IV}–Ph species drives the migration of the phenyl group to the porphyrin nitrogens because the iron is returned in the process to the ferrous oxidation state (Guilard & Kadish, 1988). The observation that the 3,5-dichlorophenyl–iron complex is stable within the cytochrome P450 active site despite prolonged treatment with ferricyanide suggests that the electron-withdrawing chlorine atoms raise the oxidation potential of the complex to the point that it cannot be oxidized in situ. A simple steric rationale for the stability of the complex is untenable because the 3,5-dimethylphenyl group, with two comparably sized but electron-releasing substituents, readily undergoes the iron–nitrogen shift under the same conditions. Shift of the 3,5-dichlorophenyl moiety when the complex is extracted into acidic solution under aerobic conditions does not conflict with this interpretation because the shift takes place under quite different experimental conditions. The interpretation is supported by preliminary evidence indicating that the phenyl–iron complexes of proteins with imidazole (electron-poor) rather than thiolate (electron-rich) heme ligands, including myoglobin (Swanson & Ortiz de Montellano, 1991), indoleamine 2,3-dioxygenase (unpublished data), and prostaglandin synthase (unpublished data), are stable to ferricyanide.

In sum, the aryl shift method in conjunction with the use of aryl moieties of diverse structure is a powerful tool for the analysis of cytochrome P450 active-site topologies. Its use in the present study has provided refined models of the active sites of cytochromes P4502B1 and 2B2 (Figure 5). Extensions of the method now in development should make possible identification in the future of the amino acids responsible for the topological features defined in this study.

REFERENCES

Black, S. D., & Coon, M. J. (1986) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 161–216, Plenum Press, New York.

Delaforge, M., Battioni, P., Mahy, J. P., & Mansuy, D. (1986) *Chem.-Biol. Interact. 60*, 101–114.

Edwards, R. J., Murray, B. P., Boobis, A. R., & Davies, D. S. (1989) *Biochemistry 28*, 3762–3770.

Gonzalez, F. J. (1990) *Pharmacol. Ther. 45*, 1–38.

Guengerich, F. P. (1987) in *Mammalian Cytochromes P-450* (Guengerich, F. P., Ed.) Vol. 1, pp 1–54, CRC Press, Boca Raton, FL.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., & Kaminsky, L. S. (1982) *Biochemistry 21*, 6019–6030.

Guilard, R., & Kadish, K. M. (1988) *Chem. Rev. 88*, 1121–1146.

Hunsberger, I. M., Shaw, E. R., Fugger, J., Ketcham, R., & Lednicer, D. (1956) *J. Org. Chem. 21*, 394–399.

Jefcoate, C. R. (1986) in *Cytochrome* P450: *Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 387–428, Plenum Press, New York.

Jonen, H. G., Werringloer, J., Prough, R. A., & Estabrook, R. W. (1982) *J. Biol. Chem. 257*, 4404–4411.

Kunze, K. L., & Ortiz de Montellano, P. R. (1981) *J. Am. Chem. Soc. 103*, 4225–4230.

Kunze, K. L., Mangold, B. L. K., Wheeler, C., Beilan, H. S., & Ortiz de Montellano, P. R. (1983) *J. Biol. Chem. 258*, 4202–4207.

Nebert, D. W., Nelson, D. R., Coon, M. J., Estabrook, R. W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R., & Waxman, D. J. (1991) *DNA Cell Biol. 10*, 1–14.

Nelson, S. D. (1987) in *Mammalian Cytochromes P-450* (Guengerich, F. P., Ed.) Vol. 2, pp 19–80, CRC Press, Boca Raton, FL.

Nelson, D. R., & Strobel, H. W. (1988) *J. Biol. Chem. 263*, 6038–6050.

Nelson, D. R., & Strobel, H. W. (1989) *Biochemistry 28*, 656–660.

Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370–2378.

Ortiz de Montellano, P. R., Beilan, H. S., & Kunze, K. L. (1981) *J. Biol. Chem. 256*, 6708–6713.

Poulos, T. L. (1988) *Pharmacol. Res. 5*, 67–75.

Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195*, 687–700.

Raag, R., Swanson, B. A., Poulos, T. L., & Ortiz de Montellano, P. R. (1990) *Biochemistry 29*, 8119–8126.

Ringe, D., Petsko, G. A., Kerr, D. E., & Ortiz de Montellano, P. R. (1984) *Biochemistry, 23*, 2–4.

Ryan, D. E., Thomas, P. E., & Levin, R. (1982) *Arch. Biochem. Biophys. 216*, 272–288.

Stanley, W. M., & Adams, R. (1930) *J. Am. Chem. Soc. 52*, 1200–1205.

Suwa, Y., Mizukami, Y., Sogawa, K., & Fujii-Kuriyama, Y. (1985) *J. Biol. Chem. 260*, 7980–7984.

Swanson, B. A., & Ortiz de Montellano, P. R. (1991) *J. Am. Chem. Soc. 113*, 8146–8153.

Swanson, B. A., Dutton, D. R., Lunetta, J. M., Yang, C. S., & Ortiz de Montellano, P. R. (1991) *J. Biol. Chem. 266*, 19258–19264.

Swanson, B. A., Halpert, J. R., Bornheim, L. M., & Ortiz de Montellano, P. R. (1992) *Arch. Biochem. Biophys. 292*, 42–46.

Tretiakov, V. E., Degtyarenko, K. N., Uvarov, V. Y., & Archakov, A. I. (1989) *Arch. Biochem. Biophys. 275*, 429–439.

Tuck, S. F., Peterson, J. A., & Ortiz de Montellano, P. R. (1992) *J. Biol. Chem. 267*, 5614–5620.

Wood, A. W., Ryan, D. E., Thomas, P. E., & Levin, W. (1983) *J. Biol. Chem. 258*, 8839–8847.