Mutagenesis at a Highly Conserved Phenylalanine in Cytochrome P450 2E1 Affects Heme Incorporation and Catalytic Activity†

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ABSTRACT: The phenylalanine corresponding to Phe-429 of rabbit cytochrome P450 2E1 is 1 of approximately 10 highly conserved residues in this superfamily of over 200 sequenced enzymes. This nearly invariant residue has been postulated to be involved in electron transfer between the heme of cytochrome $P450_{cam}$ and its redox partners [Stayton, P. S., Poulos, T. L., & Sligar, S. G. (1989) *Biochemistry 28*, 8201–8205]. To test this hypothesis, oligonucleotide-directed mutagenesis was used to replace this amino acid in rabbit P450 2E1 with aspartate, arginine, leucine, tryptophan, or tyrosine, and the mutant proteins were expressed in *Escherichia coli*. Although immunoblot analysis of whole cell lysates demonstrated that all P450 proteins (mutants and wild-type) were equally well expressed on a per cell basis, in solubilized membranes only the tryptophan and tyrosine mutants yielded ferrous–CO difference spectra characteristic of P450. The specific content (nanomoles per milligram of membrane protein) and yield per liter of the Trp mutant holoenzyme were approximately one-third those of the native enzyme, suggesting that heme incorporation was hindered by tryptophan at this position, whereas the specific content and yield per liter of the Tyr mutant were significantly greater than those of the native preparation. The stability of the Trp and Tyr mutants, as judged by thermal denaturation studies, was not different from that of the native enzyme. The Trp mutant had 38% of the aniline hydroxylase activity, 25% of the *p*-nitrophenol hydroxylase activity, and 39% of the *N*-nitrosodimethylamine demethylase activity of the native enzyme, demonstrating that this substitution also decreased catalytic activity. In contrast, substitution of tyrosine at this position had no effect on these activities. The K_m value of aniline was not significantly affected by either substitution, suggesting that these two mutations caused minimal perturbation to the substrate binding site. NADPH oxidation in both the presence and absence of substrate was significantly reduced with the tryptophan mutant, suggesting that electron flow between the reductase and P450 was impaired by this mutation. These results indicate that substitutions at this position can alter electron flow between the reductase and P450 and that this may be the mechanism by which the catalytic rate is reduced with the tryptophan mutant.

In contrast to typical cytochromes, the heme of cytochrome P450 is not directly accessible from the surface of the protein, and thus the mechanism by which electrons are transferred from a redox partner into the heme to initiate catalysis is a fundamental question for this enzyme. The most likely point of transfer is across what is termed the proximal surface, where the heme edge is within about 8 Å of the surface (Poulos et al., 1985; Ravichandran et al., 1993). Indeed, site-directed mutagenesis studies have demonstrated that several basic amino acids on this surface are involved in the electrostatic interaction of $P450_{cam}$ with its electron-transfer partner putidaredoxin (Stayton & Sligar, 1990).

The phenylalanine that corresponds to Phe-429 of rabbit cytochrome P450 2E1 is 1 of approximately 10 invariant or nearly invariant residues in this superfamily of over 200 sequenced enzymes (Nelson & Strobel, 1988). It is positioned at the beginning of the heme-binding peptide characteristic of all P450 enzymes, seven residues upstream of the cysteine that provides the thiolate ligand to the heme iron (Figure 1). This highly conserved residue is situated on the proximal surface of $P450_{cam}$, where its aromatic side chain may participate in electron transfer to the buried heme group, as proposed by Stayton et al. (1989). The highly conserved nature of this residue and its position at the putative electron-transfer surface argue for a critical role for this residue in the catalytic function of cytochrome P450.

rabbit P450 2E1	F	S	A	G	K	R	V	C	V	G	E	G	L	A
Subfamily 2E	F	S	·	G	K	R	V	C	·	G	E	G	L	A
Subfamily 2C	F	S	·	G	·	R	·	C	·	G	E	·	L	A
Family 2	F	S	·	G	·	·	·	C	·	G	·	·	·	A

FIGURE 1: Position of the mutagenized phenylalanine in the heme-binding segment of cytochromes P450. The conserved phenylalanine, corresponding to Phe-429 in rabbit cytochrome P450 2E1, is boxed; the invariant cysteine that contributes the thiolate ligand to the heme iron is in the center of the sequence. *Dots* indicate positions of variability within each group of sequences. Only two forms of P450 are known to lack this phenylalanine: In P450 10, from a pond snail, it is replaced by a tryptophan (Teunissen et al., 1992), and in P450 74, from flaxseed, it is replaced by a proline (Song et al., 1993).

To test the hypothesis that this phenylalanine is involved in electron transfer, site-directed mutagenesis was used to replace this residue in rabbit cytochrome P450 2E1 with five amino acids having differing degrees of similarity to phenylalanine, and chosen to yield a range of effects on electron transfer: charged residues (aspartate and arginine) likely to be most disruptive; leucine, which will preserve the hydrophobicity but not the aromaticity; and tryptophan and tyrosine, providing both hydrophobicity and aromaticity, the latter characteristic perhaps most critical to electron transfer. The stability and catalytic activity of the resulting mutated enzymes were assessed following their expression in *Escherichia coli*.

EXPERIMENTAL PROCEDURES

Expression Vector and Mutagenesis. The bacterial expression vector containing the rabbit cytochrome P450 2E1

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cDNA was modified from that used previously (Larson et al., 1991a) by the addition of the bacteriophage T7 gene 10 translational enhancer, as described (Pernecky et al., 1993), followed by the removal of several unwanted vector restriction sites, to yield pJL2/3a. Mutagenesis at phenylalanine-429 was performed with the use of the polymerase chain reaction (PCR)[1] and an oligonucleotide encompassing an upstream *Eco*RI site and five mutagenic primers spanning the Phe-429 codon and a downstream *Mlu*I site. Oligonucleotides were synthesized at the University of Kentucky Macromolecular Structural Analysis Facility. The 120-bp PCR products generated with *Pfu* polymerase (Stratagene) were digested with *Eco*RI and *Mlu*I and cloned into an M13mp18 vector containing the larger *Bam*HI–*Hin*dIII fragment of the P450 2E1 cDNA, and mutants were confirmed by DNA sequence determination across the entire 120-bp segment. The mutagenized *Bam*HI–*Hin*dIII fragments were then substituted for the wild-type segment in pJL2/3a.

Expression, Preparation, and Analysis of Mutant Enzymes. Up to 9 L of Luria–Bertani medium was inoculated (1:100) with the desired clone in *E. coli* strain XL1 Blue (Stratagene) from an overnight culture, and incubated at 37 °C with vigorous shaking until the OD_{600} reached approximately 0.8. Isopropyl thiogalactoside (IPTG) was added to 1 mM final concentration and 4-methylpyrazole, a high-affinity ligand for P450 2E1, to 5 μM, and incubation was continued for 4 h. Cells were harvested and membranes were prepared and solubilized as previously described (Larson et al., 1991a). P450 content was determined in solubilized membranes from the reduced CO difference spectrum in the presence of 1 μM methyl viologen. Apoenzyme expression and subcellular distribution were determined by SDS–polyacrylamide gel electrophoresis followed by immunoblot analysis with the use of polyclonal antibody to rabbit P450 2E1 and an alkaline phosphatase-conjugated secondary antibody. Protein concentration was determined with the BCA assay (Pierce).

Preparation of Cytochrome P450 Reductase. Rat cytochrome P450 reductase was expressed from pOR262 (Shen et al., 1989) and prepared and used as a solubilized membrane fraction, as described above for P450. The content was determined from the rate of cytochrome *c* reduction (Vermilion & Coon, 1978). No difference was found between the use of the bacterially expressed rat reductase and purified reductase from rabbit liver.

Activity Measurements. Incubations contained 50 pmol of P450 and 150 pmol of P450 reductase, added as solubilized cell membranes from which the octyl glucoside detergent had been removed by ultrafiltration in Microcon-30 concentrators (Amicon). The P450- and reductase-containing membranes were mixed before detergent removal; dialysis buffer contained 100 mM potassium phosphate, 1 mM EDTA, 20% glycerol, and 50 μM 4-methylpyrazole, at pH 7.4. The final concentration of 4-methylpyrazole was typically 1–2 μM in the incubations, and was not inhibitory with saturating substrate concentrations; it was omitted from the dialysis buffer without detriment to further reduce its concentration with preparations used for the kinetic analysis of aniline hydroxylation. Incubations were carried out in 50 mM potassium phosphate buffer, pH 7.6 (aniline); in 100 mM potassium phosphate, pH 6.8, and 1 mM ascorbate (*p*-nitrophenol); or in 50 mM potassium phosphate, pH 6.8 (*N*-nitrosodimethylamine, NDMA), in a total volume of 1 mL. Substrate concentrations were as follows: aniline, 2.5 mM, except as indicated with the kinetic analysis studies; *p*-nitrophenol, 0.1 mM; NDMA, 10 mM. Reactions were initiated after a 5-min incubation at 37 °C by the addition of NADPH to 1 mM, carried out in duplicate for 10 and 20 min, and stopped with 0.3 or 0.5 mL of 20% trichloroacetic acid (aniline and NDMA assays, respectively) or 0.5 mL of 35% perchloric acid (*p*-nitrophenol assay), and the protein precipitates were pelleted in a clinical centrifuge. The formation of *p*-aminophenol from aniline and of 4-nitrocatechol from *p*-nitrophenol was determined spectrally as previously described (Larson et al., 1991b); formaldehyde formation from NDMA was determined with the Nash reagent (Nash, 1953) and measured spectrally after extraction into isobutanol.

Determination of NADPH Oxidation. Incubations were as described above for aniline hydroxylation, but were initiated with the addition of NADPH to 0.5 mM, and carried out for 20 min. Reactions were stopped by transferring the tubes to a 65 °C water bath for 10 min; NADPH oxidation was determined from the decrease in absorbance at 340 nm, with use of the extinction coefficient for NADPH of 6.22 mM^{-1}. *p*-Aminophenol formation from aniline was then determined as described above. The contribution of *E. coli* enzymes in the solubilized membrane preparations to NADPH oxidation was determined in separate incubations of either P450-containing or reductase-containing membranes, and these rates were subtracted from the rate obtained with the combined membranes. The values reported thus represent the rate of NADPH oxidation specifically attributable to electron flow from the reductase through cytochrome P450 (approximately 40% of the total NADPH oxidation for the native and Tyr samples, and 20% of the total for the Trp sample), with or without substrate present.

RESULTS

The expression of the five P450 mutants in *Escherichia coli* was evaluated by immunoblot analysis, as shown in Figure 2. All five mutant proteins were equally well expressed on a per-cell basis, and at a level equivalent to that of the native, or wild-type, enzyme. Subcellular fractionation revealed that the mutations did not affect the partitioning of the protein into the *E. coli* membrane fraction, with the majority of each mutant protein being membrane-bound, as is found with the wild-type enzyme.

Although all mutant apoproteins were well expressed, only membranes containing the tryptophan and tyrosine mutants yielded measurable cytochrome P450 holoenzyme, as indicated by a spectral peak at 450 nm when reduced and complexed with carbon monoxide. Because bacterial membranes contain several cytochromes that bind CO and yield an absorbance peak at 420 nm (Porter & Larson, 1991), it was not possible to determine if the aspartate, arginine, and leucine mutants contained heme bound in a nonnative configuration, otherwise revealed by an absorbance maximum at 420 nm. The yield and specific content of the tryptophan mutant were one-third those of the native enzyme (Table 1), suggesting that heme incorporation was hindered by tryptophan at this position, given similar levels of apoenzyme expression (as determined by immunoblotting). In contrast, the yield of the tyrosine mutant was somewhat greater than that of native form.

The stability of the Trp and Tyr mutant enzymes was assessed through the generation of thermal denaturation curves, with comparison to that obtained with the native enzyme. As shown in Figure 3, the extent of denaturation during incubation at 37, 42, and 47 °C for 30 min, measured as the loss of ability to form a ferrous–CO complex with an

[1] Abbreviations: PCR, polymerase chain reaction; IPTG, isopropyl thiogalactoside; NDMA, *N*-nitrosodimethylamine.

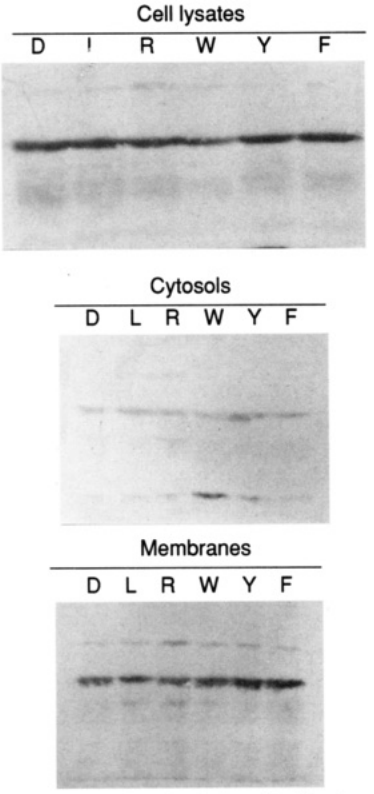

FIGURE 2: Expression and subcellular distribution of native and mutant P450 apoproteins in *Escherichia coli*. Whole cell lysates or the cytosolic or membrane fractions were prepared, fractionated by SDS–polyacrylamide gel electrophoresis, and electroblotted to nitrocellulose, and P450 protein was immunochemically detected. The mutant and wild-type enzymes are indicated by the single-letter amino acid code. Equal amounts of cells or protein (10 μg) were loaded.

Table 1: Expression of Native and Mutant P450 2E1 in *E. coli*[a]

P450	nmol of P450/L of culture	nmol/mg of solubilized membrane protein
native	18.3	4.3
Trp mutant	6.2	1.2
Tyr mutant	30.8	5.7

[a] Results are the average of 3–5 preparations.

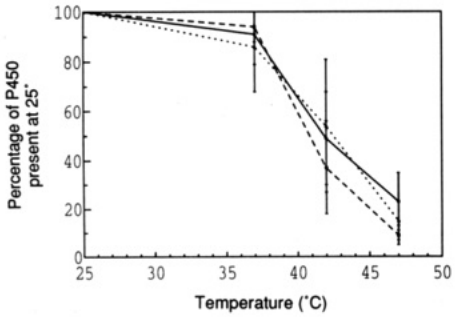

FIGURE 3: Thermal denaturation curves. Solubilized membrane preparations (2 mg/mL protein, 1–2 nmol of P450) were incubated at the indicated temperature for 30 min, then reduced with sodium hydrosulfite and bubbled with carbon monoxide, and the difference spectra were recorded. The peak height at 450 nm is plotted as a percentage of the height obtained at 25 °C for the native (—), Trp (- - -), and Tyr (·•·) preparations. Error bars indicate the standard deviation of the mean of two to five determinations.

absorbance maximum at 450 nm, was not different among the three enzymes. Similar results were obtained following sequential incubation of the reduced, CO-complexed enzymes at progressively elevated temperatures (data not shown). These results indicate that the Trp and Tyr substitutions do not destabilize the enzyme, although, as noted above, the tryptophan substitution may impede the incorporation of heme into the newly synthesized protein.

Table 2: Catalytic Activities of Native and Mutant P450 2E1 Enzymes

P450	act. [nmol of product formed min^{-1} (nmol of P450)$^{-1}$][a] for substrate		
	aniline	*p*-nitrophenol	NDMA
native	29.1 ± 6.6 (100)	9.4 ± 1.0 (100)	9.3 ± 2.5 (100)
Trp mutant	11.1 ± 1.9[b] (38)	2.4 ± 0.6[b] (25)	3.6 ± 0.6[b] (39)
Tyr mutant	34.2 ± 8.7 (118)	7.8 ± 1.4 (83)	7.7 ± 3.0 (83)

[a] Means ± standard deviations of four determinations; values in parentheses indicate activity as a percentage of that of the native (wild-type) form. [b] Significantly different from the native enzyme value ($p < 0.05$), ANOVA with Dunnett's multiple comparisons test.

The activity of the tryptophan and tyrosine mutants toward three substrates for P450 2E1 is shown in Table 2. No aniline hydroxylase activity was obtained with the aspartate, arginine, and leucine mutants, consistent with their lack of a ferrous–CO difference peak at 450 nm; the other substrates were subsequently not assayed with these mutants. Turnover numbers for the native enzyme with aniline and *p*-nitrophenol are similar to those obtained previously with bacterially expressed P450 2E1 (Larson et al., 1991b), and the activity with NDMA is slightly greater than that obtained with the enzyme purified from rabbit liver (Ding et al., 1991). These substrates are not metabolized in *E. coli* membrane preparations from cells lacking the P450 expression vector, and activity is fully dependent upon the addition of cytochrome P450 reductase and NADPH to the samples. As product formation with each substrate is equivalent to that obtained with the purified liver enzyme, further metabolism of the products in bacterial membranes appears unlikely, but was not examined. With the tryptophan mutant, turnover of all three substrates was reduced to 25–39% of that of the native enzyme; in contrast, the activity of the tyrosine mutant was not significantly different from that of the wild-type enzyme. To further ensure that the tryptophan mutant was not simply less stable than the wild-type enzyme, and that the lower activity of this mutant was not due to loss of activity during incubation at 37 °C, aniline hydroxylase reactions were carried out at 25 °C. Product formation with all three enzymes (native, Trp, and Tyr mutants) was reduced by approximately 85% from that obtained with each at 37 °C (data not shown); the activity of the tryptophan mutant was still approximately one-third that of the native enzyme. This concordantly lower activity at both 37 and 25 °C also argues against enzyme inactivation during turnover as a explanation for the reduced activity of the tryptophan mutant, a conclusion further supported by the linear reaction rates with time for all substrates and enzymes. Overall, these results indicate that the tryptophan mutant is inherently less active than the wild-type and tyrosine mutant P450 enzymes.

To determine the mechanism by which the tryptophan mutation was affecting catalysis, kinetic studies were carried out on aniline hydroxylation. A substrate concentration *versus* velocity plot with the native enzyme and the tryptophan and tyrosine mutants is shown in Figure 4, and the derived kinetic constants are presented in Table 3. With both mutants, the apparent K_m of aniline was slightly increased over that of the native enzyme; the K_{cat} values for all three enzymes were slightly greater than the rates obtained experimentally with saturating aniline concentrations (Table 2). The relatively

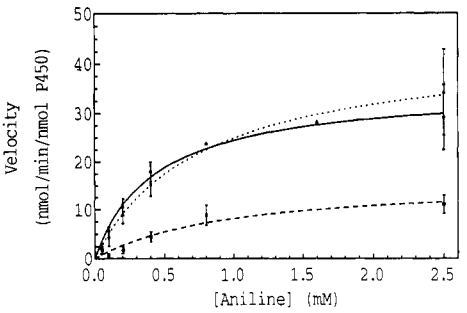

FIGURE 4: Substrate concentration *versus* velocity curves for aniline hydroxylation. *p*-Aminophenol formation from aniline in the presence of saturating levels of cytochrome P450 reductase and NADPH is plotted against substrate concentration. The data points obtained with each preparation, native (—), Trp (- - -), and Tyr (· · ·), were fitted to the curve describing a rectangular hyperbola using the equation $y = ax/(b + x)$. Error bars indicate the standard deviation of three determinations in duplicate.

Table 3: Kinetic Constants for Aniline Hydroxylation[a]

P450	app K_m[b] (mM)	K_{cat} (min^{-1})	K_{cat}/K_m (min^{-1}·mM^{-1})
native	0.44	35.1 (100%)	80.8 (100%)
Trp	0.96	16.1 (46%)	16.7 (21%)
Tyr	0.75	43.7 (125%)	58.1 (72%)

[a] Values are derived from the fitted curves using the equation $y = ax/(b + x)$, where $a = K_{cat}$ and $b = K_m$. [b] Apparent K_m, reductase and NADPH concentrations were saturating.

Table 4: Stoichiometry of NADPH Oxidation

P450	nmol of NADPH oxidized[a]		nmol of *p*AP formed	ratio[b]
	−aniline	+aniline		
native	39	39	30	1.3:1
Trp	1	17	14	1.2:1
Tyr	38	46	33	1.4:1

[a] Nanomoles per 20-min incubation in the absence (−) or presence (+) of 2.5 mM aniline, and is equivalent to nanomoles per minute per nanomoles of P450; average of three determinations. [b] Ratio of nanomoles of NADPH oxidized in the presence of aniline to nanomoles of *p*-aminophenol (*p*AP) formed.

small changes in the apparent K_m suggest that mutations at position 429 have minimal effect on substrate binding (assuming that K_m largely reflects substrate binding affinity); moreover, a modest reduction in substrate affinity (as found here) should be overcome at high substrate concentration, such that K_{cat} should not be affected. Thus, the 54% reduction in K_{cat} with the tryptophan mutant argues that this mutation has a significant effect on the intrinsic activity of the enzyme. In contrast, substitution of tyrosine at this position appears to have little effect on the activity of the enzyme.

The possibility that the mutations were altering the coupling of NADPH oxidation to substrate metabolism was assessed by examining the stoichiometry of the two reactions, as shown in Table 4. It is well established that P450 2E1 has a high rate of electron turnover in both the presence and absence of substrate, with the generation, in part, of reactive O_2 species (Morgan et al., 1982; Gorsky et al., 1984). The coupling ratio of NADPH oxidation to aniline hydroxylation was approximately 1.3:1 for the native P450 preparation, and was not greatly different with either the tryptophan or the tyrosine mutant. However, in the absence of substrate, the rate of NADPH oxidation was significantly reduced with the tryptophan mutant, whereas the native and tyrosine preparations oxidized NADPH in the absence of substrate at rates similar to those obtained with aniline present. Thus, the coupling efficiency of the tryptophan mutant approaches 100%, but at the expense of a 50–60% reduction in substrate turnover. These results suggest that substitutions at this position can alter electron flow between the reductase and P450 and that this may be the mechanism by which the catalytic rate is reduced with the tryptophan mutant.

DISCUSSION

In the present study, a variety of amino acids were substituted for the nearly invariant phenylalanine in P450 2E1, and the effect of these changes on the stability and catalytic activity of enzyme was investigated. One other group has reported the effect of mutagenesis at this highly conserved phenylalanine: Shimizu et al. (1988) replaced Phe-449 of rat cytochrome P450 1A2 with tyrosine and found that, although apoenzyme was present at a level equivalent to that of the wild-type enzyme, as determined by immunoblot analysis of yeast cell lysates, the mutant holoenzyme was present at only 20% of the level of the native holoenzyme, and the ferrous–CO absorbance maximum was shifted from 448 to 445 nm. The mutant enzyme also lacked 17β-estradiol hydroxylase activity and was reported to be unstable. These results are in contrast to the present findings, in which substitution of tyrosine for the conserved phenylalanine in P450 2E1 had no significant effect on the spectral properties or catalytic activity of the enzyme, and the tyrosine mutant was in fact more efficiently expressed in *E. coli* than the wild-type enzyme. Stephen Sligar's group originally suggested that this phenylalanine might participate in electron transfer between putidaredoxin and the heme of $P450_{cam}$ (Stayton et al., 1989); in an effort to test this hypothesis, however, they found that substitution of this phenylalanine in $P450_{cam}$ with leucine or isoleucine prevented proper heme incorporation and did not yield functional enzyme (Stephen G. Sligar, personal communication). These results are consistent with the present finding that substitution of leucine (and also aspartate and arginine) at this position in P450 2E1 prevents the formation of intact P450 holoenzyme, as indicated by the lack of an absorbance maximum at 450 nm when the enzyme is reduced and complexed with carbon monoxide.

It thus appears that an aromatic residue is required at position 429 for proper heme incorporation into P450 2E1. The substitution of aspartate, arginine, or leucine did not yield intact P450 holoenzyme, whereas the tryptophan and tyrosine mutations yielded catalytically active enzyme that could be readily detected as the ferrous CO–heme complex. In addition, holoenzyme formation (but not stability) appears to be quite sensitive to the nature of the aromatic side chain at this position, in that the yield of the tryptophan mutant holoenzyme was significantly less than that of the native and tyrosine mutant enzymes. Once formed, however, the tryptophan and tyrosine mutants were no more sensitive to thermal denaturation than the wild-type enzyme (Figure 3). These results suggest that an aromatic residue is necessary at this position in the folding pathway leading to heme incorporation, in addition to any part played by this residue in electron transfer in the mature enzyme. Indeed, it may not be possible to accurately assess the role of this amino acid in electron transfer, if an aromatic residue is obligatory at this position for holoenzyme formation; the substitution of a nonaromatic residue, which might more effectively impede electron transfer, would prevent the incorporation of heme into the enzyme.[2]

[2] It should be noted that expression in *E. coli* may not accurately reflect expression in mammalian cells, as has been shown with a shortened form of cytochrome P450 17 (Sagara et al., 1993).

An intimate interaction between the heme and the amino acid at this position is in accord with the two known three-dimensional structures of P450: $P450_{cam}$ from *Pseudomonas putida* and $P450_{BM-3}$ from *Bacillus megaterium*, in which the phenylalanine at this position is located at the surface of the proteins and is situated so as to shield the heme from the surrounding solvent (Poulos et al., 1985, 1987; Ravichandran et al., 1993). Because this surface is also thought to be the docking surface for cytochrome P450 with its electron-transfer partners (*i.e.*, a ferredoxin or cytochrome P450 reductase), this aromatic residue is an attractive candidate for a role in electron transfer between the redox centers of the two proteins. The tryptophan and tyrosine mutants were designed to be conservative substitutions for this phenylalanine, and were not expected to strongly disrupt electron transfer, as both maintain the aromaticity at this position. Thus, the lack of effect of the tyrosine mutation on catalytic activity and the modest reduction in activity with the tryptophan mutation are consistent with this prediction. Nonetheless, the similar extent of reduction in turnover numbers for the tryptophan mutant with three structurally diverse substrates, and the small increase in the K_m of aniline, argues that this substitution has a greater effect on the intrinsic activity of the enzyme than on the binding of substrate. The significant reduction in NADPH oxidation with the tryptophan mutant, both in the presence and in the absence of substrate, suggests that electron flow between the reductase and P450 is impaired; whether this is due to a reduction in electron-transfer efficiency, or reduced affinity or altered binding of the two proteins, remains to be determined. That tyrosine apparently can replace phenylalanine at this position without obvious detrimental effect raises the question as to why this substitution is not observed in the over 200 P450 sequences now known.

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