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Cumene Hydroperoxide-Supported Demethylation Reactions Catalyzed by Cytochrome P450 2B4 Lacking the NH_2-Terminal Sequence

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Catalytic activities of cytochrome P450 2B4 lacking NH_2-terminal amino acids 2-27 (wt Δ2B4) and that of truncated 2B4 containing a Pro to Ser mutation at position 221 were examined in a system supported by cumene hydroperoxide. Demethylation activities of either truncated 2B4 with N-methylaniline, N,N-dimethylaniline, and *d*-benzphetamine were lower than those of liver microsomal 2B4, whereas the rate of 1-phenylethanol oxidation to acetophenone catalyzed by liver microsomal and truncated 2B4 enzymes was nearly the same. The K_m and V_{max} values for cumene hydroperoxide in the demethylation of N-methylaniline by wt Δ2B4 were 20% and 28%, respectively, of those obtained for 2B4. The reaction with wt Δ2B4 displayed a lesser dependence on phospholipid than did that with 2B4, and a complex relationship between activity and substrate concentration. The results suggest that the NH_2-terminal region contributes to interaction of oxidant, substrate, and phospholipid in cumene hydroperoxide-supported reactions catalyzed by cytochrome P450 2B4.

Key Words: **cytochrome P450; hydroperoxides; heterologous expression.**

Cytochrome P450 monooxygenases comprise a superfamily of versatile catalysts that bring about the hydroxylation of an array of structurally dissimilar xenobiotics and endogenous steroids and fatty acids (1,2). The eukaryotic monooxygenase system consists of the heme-containing P450, the flavoprotein NADPH-cytochrome P450 reductase, which provides reducing equivalents for oxygen activation, and phospholipid, which is required for optimal activity (1).

A number of mammalian P450 cytochromes lacking the NH_2-terminal region have been expressed in heterologous (4-11) and cell-free (12) systems in an attempt to understand the role of the noncleavable signal peptide in membrane binding and catalytic function. Full-length P450 2E1 and 2E1 lacking amino acids 3-29 are predominantly localized in the membrane fraction when expressed in *Escherichia coli* (7). On the other hand, removal of amino acids 2-27 from P450 2B4 results in localization of the truncated protein predominantly in the cytosolic compartment of *E. coli* (9), and deletion of amino acids 3-20 from P450 2C3 yields an expressed protein that is a soluble dimer (10). As with membrane binding, catalytic activity of the shortened P450s in the reconstituted system varies with the particular P450 isoform. P450 2E1 without amino acids 2-29 has the same catalytic activity as intact 2E1 purified from rabbit liver microsomes (13), whereas 2B4 lacking residues 2-27 possesses lower activity than full-length 2B4 purified from liver microsomes of phenobarbital-treated rabbits (14). The results of a few studies suggest that optimal complex formation between P450, P450 reductase, lipid, and/or substrate may be impaired by removal of the NH_2-terminal region. For example, the catalytic activity of truncated 2B4 with some substrates can be increased to equal that of 2B4 by allowing the components of the reconstituted system to interact for 30 min prior to determination of catalytic activity (15). A facilitory role for the NH_2-terminal region of P450 in interaction with the reductase is suggested by the greater molar ratio of reductase to P450 required for maximal catalytic activity with truncated P450 52A3 than with the intact enzyme (16). A similar conclusion is derived from the demonstration of a diminished rate of reduction of 2B4 lacking amino acids 2-27 under steady state conditions (17).

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Abbreviations used: CHP, cumene hydroperoxide; P450, cytochrome P450 (see Ref. 3 for updated P450 nomenclature); dilauroyl-GPC, dilauroyl-glyceryl-3-phosphorylcholine; DMF, dimethyl formamide.

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TABLE 1

Component Requirements for CHP-Supported N-Methylaniline Demethylation by (Δ)2B4

System	HCHO formed (μM min^{-1})[c]		
	2B4	wt Δ2B4	Δ2B4:P221S
Complete[a]	24.5	11.3	11.0
-(Δ)2B4, + boiled (Δ)2B4[b]	ND	2.3	2.3
-di:12-GPC	4.0	4.8	6.3
-N-methylaniline	0.3	ND	0.5
-CHP	1.0	1.3	ND

Note. ND, none detected.

[a] P450 was at 0.25 μM, and the concentration of the other components was as indicated in Experimental Procedures.

[b] P450 preparations were boiled for 10 min.

[c] The activity of the system in the absence of P450, or for the mixture with 2% DMF but no substrate, was 0.3 nmol HCHO formed/min/mL.

The replacement of NADPH and P450 reductase by artificial oxidants such as CHP or iodosobenzene, which provide electrons and oxygen to P450, has provided insight into the nature of the active oxidant formed during catalysis without the requirement for the P450 reductase to support catalytic activity (18-20). The purpose of the present study is to compare CHP-supported substrate hydroxylation reactions by liver microsomal P450 2B4 and P450 2B4 lacking the NH_2-terminal region.

EXPERIMENTAL PROCEDURES

Purification of P450 2B4 preparations. Rabbit P450 2B4 was purified from liver microsomes of phenobarbital-treated rabbits according to established methods (21). The recombinant P450 2B4 without amino acid residues 2-27 is reported to contain a Ser at position 221 (Dr. Peter Hlavica, personal communication) and is designated in the present study as Δ2B4:P221S. The mutation that gives rise to Pro-221 was effected by oligonucleotide-directed mutagenesis to produce a truncated P450 2B4 that is identical in sequence to the wild-type enzyme purified from rabbit liver microsomes (22), and is designated wt Δ2B4. Recombinant proteins were purified fused to glutathione S-transferase on a GSH resin by established procedures (23). Preparations of all three proteins were generously provided by Drs. Kostas P. Vatsis and Minor J. Coon; the specific contents of the 2B4, wt Δ2B4, and Δ2B4:P221S preparations were 18.4, 15.3, and 11.6 nmol/mg protein, respectively, and were electrophoretically homogeneous. The concentration of P450 was determined from the absolute spectrum of the CO-complexed ferrous cytochrome with an absorption coefficient of 110 mM^{-1} at 451 nm (21) and the protein concentration of the preparations was determined by the bicinchoninic protein assay (Pierce).

Determination of CHP-supported catalytic activity. All reagents were obtained from Sigma and Aldrich. The concentration of CHP was determined by iodometric titration (24) and found to be within 5% of that stated by the manufacturer; the oxidant was not further purified before use. Dilauroyl-GPC was dispersed in distilled and deionized water by sonication before delivery to the reaction mixture. N-Methylaniline solutions were prepared in DMF such that the final solvent concentration was no more than 2% (v/v) in the reaction mixtures. Reaction mixtures were composed of the following unless otherwise indicated: 1.0 μM purified cytochrome P450, 90 μg dilauroyl-GPC per mL, 2 mM N-methylaniline, and 1 mM CHP in 0.25 mL. Mixtures with all the components present except CHP were equilibrated at 30°C for 3 min, and the reactions were initiated with cumene hyperperoxide and quenched after 2 min at 30°C with 50 μL of 45% trichloroacetic acid. The precipitated protein was removed by centrifugation, and formaldehyde in the supernatant fraction along with a set of standards was determined by the Nash assay (25,26). The color development in the Nash assay was maximal after a 30-min incubation at room temperature. Zero-time blanks with all the components were quenched with TCA on ice prior to addition of CHP, and the values were subtracted from those of the reaction mixtures. Acetophenone produced from oxidation of 1-phenylethanol was assayed by HPLC (27). All values are given as the mean of triplicate determinations and did not vary by more than 10% unless otherwise indicated.

Determination of P450 concentration during turnover. The reaction mixtures contained the same components as described above, but with 0.25 μM cytochrome P450. Ferrous carbonyl difference spectra were recorded immediately after the 2-min incubation with CHP (28).

RESULTS

Characterization of (Δ)2B4-catalyzed N-methylaniline demethylation supported by CHP. In earlier studies, Δ2B4 was found to have lower activity than 2B4 from liver microsomes of phenobarbital-treated rabbits regardless of the substrate examined (14). In the present study, the reductase and NADPH were replaced with CHP. The shortened P450 used in previous studies (14,23) had a sequence that coded for a Ser at position 221 (personal communication), rather than the Pro that is reported for the cDNA-encoded amino acid sequence (29) and the amino acid sequence of purified liver microsomal P450 2B4 (22). In view of this

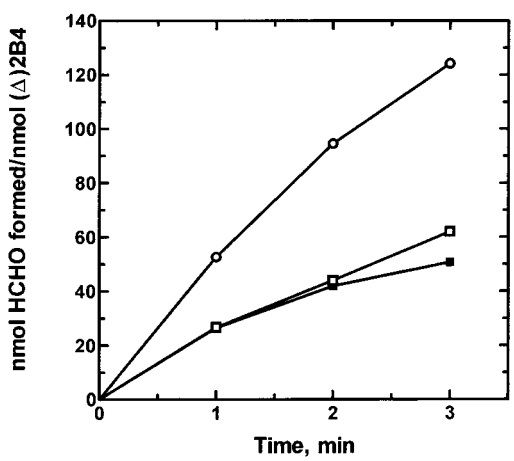

FIG. 1. Time course of (Δ)2B4-catalyzed N-methylaniline demethylation supported by CHP. The conditions were as described in Experimental Procedures, except for the inclusion of (Δ)2B4 at 0.5 μM. The time-dependent formation of formaldehyde is shown for 2B4 (open circles), wt Δ2B4 (open squares), and Δ2B4:P221S (closed squares). The values are given as the mean and standard deviation of triplicate determinations.

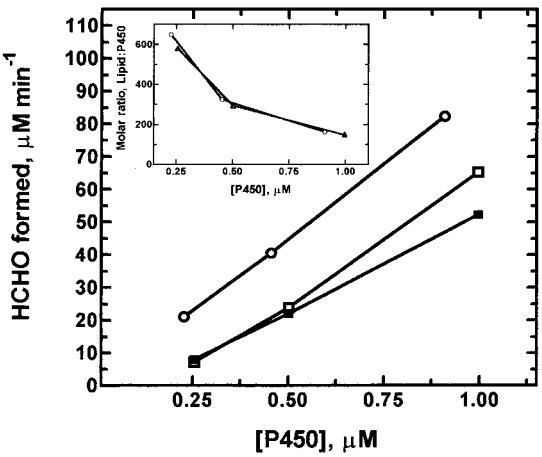

FIG. 2. CHP-supported N-methylaniline demethylation as a function of (Δ)2B4 concentration. The conditions were as described in Experimental Procedures and in the text, except that the concentration of 2B4 (open circles), wt Δ2B4 (open squares), and Δ2B4:P221S (closed squares) was varied as shown. The inset depicts the relationship between P450 concentration and the molar ratio of lipid to 2B4 (closed circles) and recombinant Δ2B4 (open triangles). The lipid was held constant at 90 μg per mL reaction mixture.

difference in sequence, this truncated mutant P450, herein designated Δ2B4:P221S, was investigated in the present study together with the wild type truncated P450 2B4, designated wt Δ2B4.

The CHP-supported demethylation of N-methylaniline by liver microsomal 2B4, wt Δ2B4 and Δ2B4: P221S is shown in Table 1. Of particular interest is the observation that the N-demethylation activity with either recombinant Δ2B4 is less than one-half that of liver microsomal 2B4. The relatively high activity of 2B4 was not due to the contribution of a component in the system other than P450 since replacement of 2B4 by heat-inactivated 2B4, or the removal of N-methylaniline, P450, or CHP resulted in activities that were less than 10% of that obtained with the complete system, in agreement with the results of an earlier study (30). On the other hand, the considerable activity observed with the boiled recombinant 2B4 enzymes suggests that components other than P450 contribute to the total activity with these preparations. Subsequently, protein preparations were dialyzed against 100 volumes of 0.1 M potassium phosphate buffer, pH 7.4, to lower the glycerol and EDTA concentrations, and P450 concentration was determined in the dialyzed preparations prior to the experiment. The CHP-supported activity of dialyzed wt Δ2B4 was found to be less than that of the nondialyzed preparation, and reconstitution of dialyzed wt Δ2B4 with glycerol and EDTA in amounts present in the nondialyzed preparation gave comparable activities (data not shown). It is not clear how these components contribute to the apparent catalytic activity of the enzyme system, but results from other laboratories suggest that oxidation of glycerol by an oxidant generated during P450 monooxygenase reactions yields formaldehyde (31), which is the product measured in the present study.

The time course of CHP-supported demethylation of N-methylaniline by 2B4 and Δ2B4 preparations is shown in Fig. 1. Formaldehyde formation with 2B4 or wt Δ2B4 is nearly linear for two minutes at 30°C. Previous studies have documented a substantial inactivation of the catalyst in reactions supported by organic peroxides (30,32-35). For this reason, the catalytic activity with CHP was determined after a 2-min time period.

Figure 2 depicts the protein concentration dependence of N-methylaniline demethylation by 2B4 and the truncated 2B4 proteins in the presence of CHP. The catalytic activity of 2B4 is directly proportional to the concentration of the hemeprotein from 0.25 to 1.0 μM. The activity of either truncated 2B4 protein is less than that of 2B4 at all protein concentrations tested. Interestingly, the turnover number of the truncated 2B4 cytochromes increases with increasing protein concentration, whereas that for 2B4 is relatively constant over the protein concentration range tested. Since the phospholipid was kept constant at 90 μg/mL, the molar ratio of lipid to P450 varied from 145:1 to about 600:1,

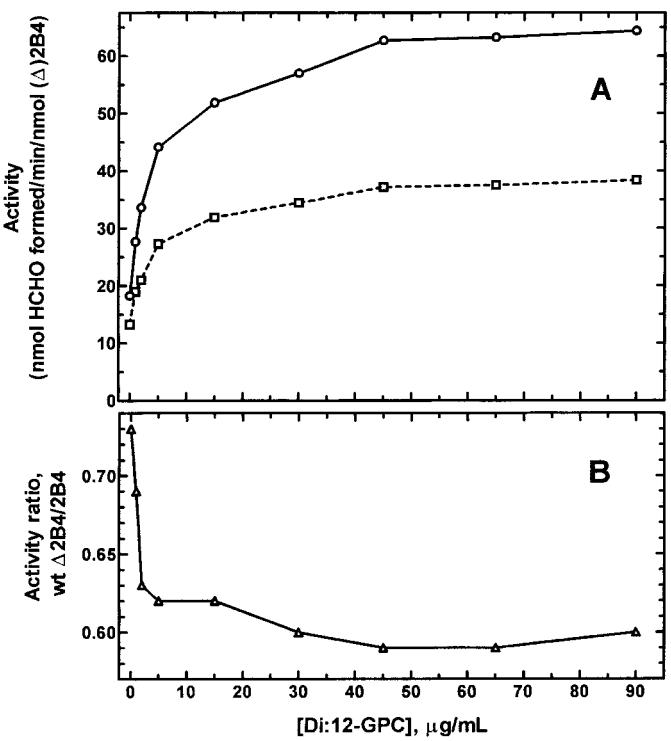

FIG. 3. CHP-supported N-methylaniline demethylation as a function of phospholipid concentration. The concentration of 2B4 (circles) and wt Δ2B4 (squares) was 0.5 μM, and the phospholipid concentration was varied as shown. The activities of the individual proteins are shown in panel A, and the ratio of the activity of wt Δ2B4 to that of 2B4 for each lipid concentration is shown in panel B.

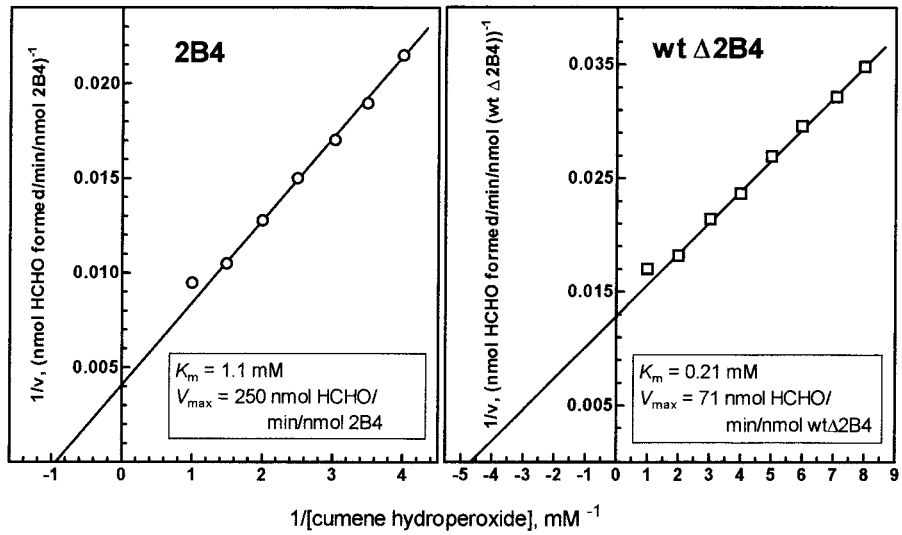

FIG. 4. Lineweaver-Burk plots for CHP in the demethylation of N-methylaniline by 2B4 (circles) and wt Δ2B4 (squares). N-methylaniline was present at 5 mM, other conditions were as described in Experimental Procedures, and the concentration of CHP was varied as shown.

as shown in the inset to Fig. 2. In other experiments not shown here, the activity of wt Δ2B4 was linear from 0.5 to 1.5 μM P450, but that of 2B4 was lower than anticipated at 1.5 μM P450, perhaps due to substrate depletion.

Lipid concentration dependence of N-methylaniline metabolism supported by CHP. Previous studies have established that different P450s require dissimilar amounts of phospholipid for maximal activity. For example, NADPH-supported benzphetamine N-demethylation by P450 2B4 is stimulated from 5- to 20-fold by dilauroyl-GPC (36,37), whereas *p*-nitrophenol hydroxylation by P450 2E1 is increased only 1.7-fold with the same lipid (13). The effect of lipid concentration on CHP-supported N-methylaniline demethylation by 2B4 and wt Δ2B4 was examined in the present study. The results shown in Fig. 3 reveal almost identical lipid concentration profiles for 2B4 and wt Δ2B4, with a half-maximal effect at less than 5 μg/mL and nearly maximal activity at 45 μg/mL, or at a molar ratio of lipid to P450 of 145:1 for either 2B4 preparation. With saturating lipid, the activity of wt Δ2B4 was 60% of that of 2B4. The activities of wt Δ2B4 and 2B4 are stimulated maximally (90 μg di:12-GPC/mL) by 2.8-fold and 3.4-fold, respectively. Interestingly, the wt Δ2B4:2B4 activity ratio decreases as the lipid concentration, or the molar ratio of lipid to P450, increases (Fig. 3B). This may provide an explanation as to why wt Δ2B4 has only about one-third the activity of 2B4 at 0.25 μM P450 when the molar ratio of lipid to protein was about 580:1 (as shown in Fig. 2), but nearly 60% the activity of 2B4 at 0.5 μM when the ratio was 113:1 to 226:1 (90 μg/mL, Fig. 3B).

Effect of CHP and N-methylaniline concentration on demethylation by 2B4 and wt Δ2B4. As shown in Fig. 4, a Lineweaver-Burk plot of the data obtained with wt Δ2B4 and varying amounts of CHP gives K_m and V_{max} values that are 20% and 28%, respectively, of those determined with 2B4. Lineweaver-Burk plots of the relationship between N-methylaniline concentration and demethylation are shown in Fig. 5. The CHP-supported reaction with 2B4 gives a linear relationship over the entire range of substrate concentrations tested. The K_m for N-methylaniline determined from

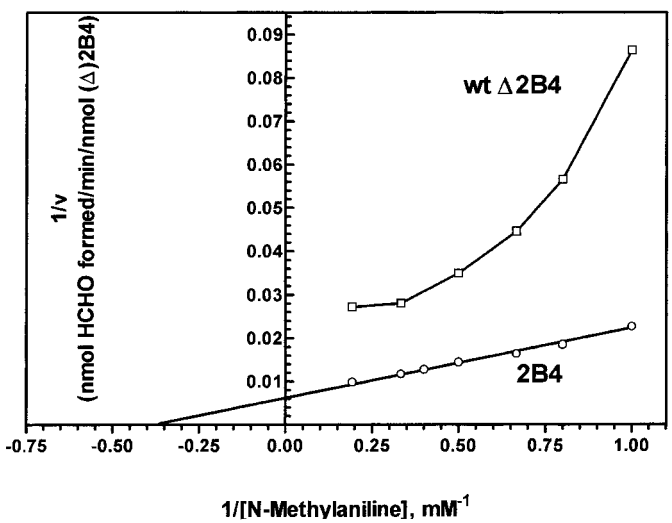

FIG. 5. Lineweaver-Burk plots for N-methylaniline in the CHP-supported demethylation catalyzed by 2B4 (circles) and wt Δ2B4 (squares). The composition of the reaction mixtures was as described in Experimental Procedures except that the concentration of N-methylaniline was varied.

TABLE 2

Effect of CHP on Carbon Monoxide Reactivity of Ferrous P450 (Δ)2B4 during Demethylation of N-Methylaniline

[N-Methylaniline] mM	CO reactivity after 2 min (% of zero time)	
	2B4	wt Δ2B4
0	18	44
2.0	21	35
5.5	49	47

this plot is 2.8 mM and the V_{max} is 165 nmol HCHO formed/min/nmol 2B4. By contrast, the double-reciprocal plot for N-methylaniline with wt Δ2B4 departs markedly from linearity, making it impossible to obtain kinetic constants. In this connection, the catalytic activity of wt Δ2B4 at 5.0 mM N-methylaniline is about 40% that of 2B4, whereas at low substrate concentration (1.0 mM), the activity of wt Δ2B4 is 25% that of 2B4. Collectively, these results suggest that the NH_2-terminal region influences the interaction of N-methylaniline and CHP with the catalyst.

Inactivation of P450 2B4 during CHP-supported demethylation of N-methylaniline. Several studies attest to the inactivation of P450 by oxidants (30,32-35). Accordingly, the carbon monoxide reactivity of ferrous P450 was examined after 2 min of incubation with CHP. As shown in Table 2, 56% of wt Δ2B4 is destroyed in the absence of substrate, whereas over 80% of 2B4 is inactivated in the same time period. The relatively lower rate of inactivation of wt Δ2B4 is reflective of its lower catalytic activity relative to 2B4. Almost threefold more 2B4 is CO-reactive at 5.5 mM N-methylaniline than in the absence of a substrate, a finding which is consistent with an earlier report of a protective effect of N-methylaniline during CHP-dependent inactivation (30). Interestingly, substrate affords no protective effect on wt Δ2B4 during CHP-supported demethylation of N-methylaniline. Collectively these experiments suggest that catalyst inactivation is not the explanation for the lower demethylation activity of truncated relative to intact 2B4.

Metabolism of various substrates by (Δ)2B4 supported by CHP. As shown in Table 3, the demethylation activities of wt Δ2B4 and Δ2B4:P221S were lower than that of 2B4 with N-methylaniline, N,N-dimethylaniline, or *d*-benzphetamine when the reaction was supported by CHP. The truncated P450s had comparable activities with N-methylaniline and benzphetamine, whereas Δ2B4:P221S had about twice the demethylation activity of wt Δ2B4 with N,N-dimethylaniline. Interestingly, the truncated P450s and 2B4 had nearly identical 1-phenylethanol oxidation activities.

DISCUSSION

The results in this study demonstrate that 2B4 without NH_2-terminal residues 2-27 has lower demethylation activity than intact 2B4 when the reactions are supported by CHP. The phospholipid requirement for the shortened 2B4 is somewhat lower than that for 2B4, although the activity of the truncated protein is less than that of 2B4 regardless of the phospholipid concentration. The mode by which phospholipid stimulates catalytic activity in peroxide-supported reactions is not currently understood. N-methylaniline and CHP interactions with the enzyme are also markedly altered in the truncated 2B4. The activity differences between microsomal 2B4 and wtΔ2B4 are not apparently due to improper folding of the recombinant P450 in *E. coli*. For example, the specificity constant, as judged by the value for k_{cat}/K_m for CHP with N-methylaniline as substrate, is not impaired by removal of the NH_2-terminal region. Moreover, the rate of formation of acetophenone from 1-phenylethanol is no different for the truncated 2B4 enzymes than for intact 2B4.

Mammalian P450 cytochromes differ from the soluble bacterial proteins heretofore crystallized in that they have a highly hydrophobic NH_2-terminal se-

TABLE 3

CHP-Supported Activity of (Δ)2B4 with Various Substrates

Substrate[a]	[Substrate], mM	[Oxidant], mM	Reaction time, min	Activity, nmol product formed/nmol P450[b]		
				2B4	wt Δ2B4	Δ2B4:P221S
N-Methylaniline	2	1.0	2	158	67 (42)	80 (51)
N,N-Dimethylaniline	2	1.0	2	132	62 (47)	110 (83)
d-Benzphetamine	1	1.0	6	21	13 (60)	14 (66)
1-Phenylethanol	10	0.5	3	89	78 (88)	77 (87)

[a] The P450 was at 1.0 and 2.0 μM in the demethylation and 1-phenylethanol oxidation reactions, respectively. The phospholipid was at 90 and 50 μg/mL for the demethylation and 1-phenylethanol oxidation reactions, respectively.

[b] The numbers in parentheses are the activities of the truncated P450s as a percent of that for 2B4.

quence, which is used for insertion of the P450 into the endoplasmic reticular membrane (38). The high degree of hydrophobicity in the NH_2-terminal region may contribute to substrate binding in view of the fact that the best substrates for P450 2B4 are themselves highly hydrophobic, and, consequently, might interact with similarly hydrophobic domains in the cytochrome to gain access to the substrate binding site (39,40). In this connection, the entrance to the substrate binding pocket in CYP102 ($P450_{BM-3}$) contains an exposed hydrophobic patch formed by nonpolar amino acid residues in the NH_2-terminal region (41).

Another interesting finding in this study was the nonlinearity of the double-reciprocal plot obtained for the relationship between N-methylaniline concentration and catalytic activity of wt Δ2B4. A deviation from linearity at relatively high substrate concentrations in Lineweaver-Burk plots of CHP-supported demethylation previously suggested the occurrence of a competition between CHP and substrate for active site binding (30). If such a competition exists, the results of the present study suggest that the competition might be amplified by removal of the NH_2-terminal region. In fact, the 5-fold lower K_m for CHP with wt Δ2B4 relative to 2B4 suggests that CHP association is kinetically favored in wt Δ2B4, and this may, in turn, result in a lowered affinity for N-methylaniline. In another report, nonlinear Lineweaver-Burk plots of microsomal progesterone 6β-hydroxylation were observed in the presence of an amino steroid (42), which was hypothesized to act as an effector of activity at a second binding site. Similarly, in the present study, the removal of the N-terminal region of 2B4 might unmask a second binding site for the N-methylaniline, which could act as an effector of demethylation activity. Further studies are needed to fully elucidate the role of the NH_2-terminal region in activation of organic peroxide and association of the substrate with cytochrome P450 in CHP-supported reactions.

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