Molecular Basis for the Differences in Lidocaine Binding and Regioselectivity of Oxidation by Cytochromes P450 2B1 and 2B2[†]

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ABSTRACT: The interactions of lidocaine (LIDO) with two closely related P450s, 2B1 and 2B2, were investigated using chimeric enzymes and single-point mutants derived from the two proteins. P450 2B1 exclusively catalyzed the *N*-deethylation of LIDO to generate monoethylglycinexylidide (MEGX) and 2B2 catalyzed both *N*-deethylation and hydroxylation reactions to generate MEGX and ω-diethylamino-2-hydroxymethyl-6-methylacetanilide. The addition of LIDO to 2B2 evoked a type I binding spectral change with a measured K_s of approximately 20 μM. The magnitude of the change in the absorbance obtained following the binding of LIDO to 2B2 was indicative of an approximately 30% switch of the heme iron to the high-spin form. In contrast, the addition of LIDO to 2B1 resulted in less than a 1% shift to the high-spin form even at LIDO concentrations as high as 10 mM. P450 2B2 exhibited a low K_m value for LIDO (62 μM), whereas 2B1 had an approximately 10-fold higher K_m value. However, the rates of LIDO oxidation by 2B1 were approximately 200-fold those exhibited by 2B2. Substitution of 2B2 residues by 2B1-derived amino acids influenced the spectral binding, regioselectivity of LIDO oxidation, and the kinetic properties of the enzyme. With the 2B2 Ala-363 to Val mutant, a complete switch of the 2B2 mutant to catalyzing only the *N*-deethylation activity was observed. The altered regioselectivity was accompanied with approximately 10-fold increases in the measured K_s, K_m, and k_{cat} values for LIDO.

The metabolism of LIDO,[1] a local anesthetic and antiarrythmic drug, has been studied extensively in experimental animals and humans (*2*−*5*). These studies have demonstrated the involvement of several forms of P450 in LIDO oxidation. The extensive metabolism observed by Boyes *et al*. (*2*) made LIDO a good example of a drug that would be completely eliminated following first-pass metabolism by the liver. The metabolic pathway shown in Figure 1 describes the possible routes for LIDO oxidation by rat liver microsomes. LIDO is both *N*-deethylated to produce MEGX and GX and hydroxylated to produce 3-OH-LID and Me-OH-LID. These metabolites possess pharmacological activities similar to those observed with LIDO and may be further oxidized following several interconnecting pathways to generate 2,6-xylidine (*4*). Although not directly implicating lidocaine as a human carcinogen, the formation of 2,6-xylidine in humans may be significant since it has been shown to be carcinogenic in rats (*6*).

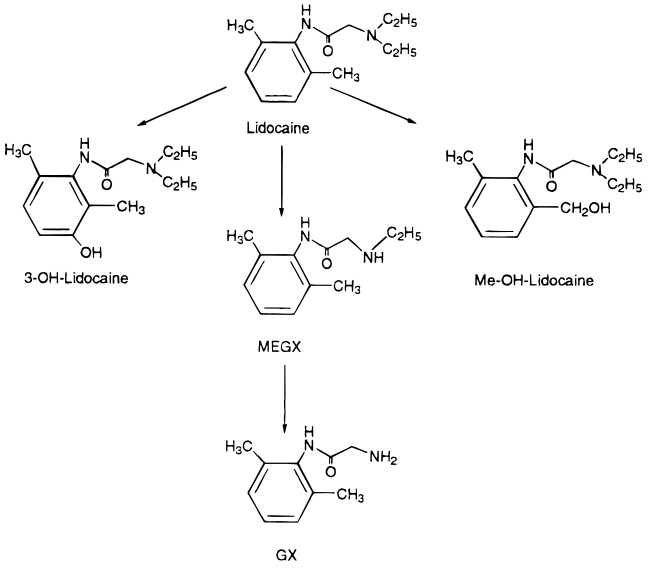

FIGURE 1: Major pathways for the metabolism of LIDO (*8*).

Initial studies using different inducers of P450 were aimed at identifying the specific forms of P450 responsible for the various metabolic pathways of LIDO (*7*, *8*). These experiments have shown enhancement of MEGX and Me-OH-LID formation following PB treatment of rats, suggesting the possible involvement of P450s 2B1 and 2B2 in the formation

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[1] Abbreviations: LIDO, lidocaine; P450, cytochrome P450 nomenclature from Nelson *et al*. (*1*); b_5, cytochrome b_5 (EC 4.4.2 group); MEGX, monoethylglycinexylidide; GX, glycinexylidide; Me-OH-LID, ω-diethylamino-2-hydroxymethyl-6-methylacetanilide; 3-OH-LID, 3-hydroxy-ω-diethylamino-2,6-dimethylacetanilide; PB, phenobarbital; δ-ALA, δ-amino levulinic acid; DLPC, L-α-phosphatidylcholine, dilauroyl (C12:0); IPTG, isopropylthio-β-D-galactoside.

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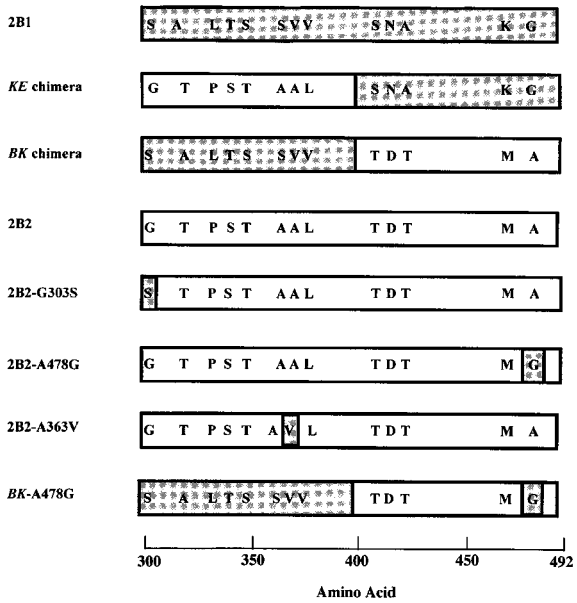

FIGURE 2: Schematic diagram representing the recombinant proteins used for these studies. Shaded boxes represent the protein regions derived from 2B1 whereas the clear boxes are those regions derived from 2B2. Amino acid differences between the two proteins are indicated by the one-letter amino acid abbreviations. The first 300 amino acids are not represented in the figure since there are no amino acid differences between the two proteins within that region.

of these products. Treatment of the rats with 3-methylcholanthrene enhanced production of 3-OH-LID, suggesting the involvement of P450s 1A1 and 1A2. Oxidation studies with the individual purified rat liver isozymes in the reconstituted system have demonstrated the different regioselectivities of the different purified isozymes for LIDO oxidation (*8*). Of particular interest were the different regioselectivities for oxidation of LIDO by the two closely related P450s 2B1 and 2B2 which have a 97% amino acid identity. The primary oxidation of LIDO by 2B1 resulted in formation of only one product, MEGX. Whereas, MEGX and Me-OH-LID were formed at a ratio of 3:1, respectively, following LIDO oxidation by 2B2 (*8*). Additionally, the overall rates for the oxidation of LIDO by 2B1 were at least 50-fold higher than those exhibited by 2B2, in accordance with known catalytic properties of the two cytochromes toward almost all substrates (*9*−*12*). Therefore, the strikingly different regioselectivities and rates of turnover exhibited by the two enzymes toward LIDO indicate that LIDO oxidation may be a useful tool to investigate the roles played by the sequence differences in the catalytic properties of each enzyme.

In this study, we have used *Escherichia coli*-expression systems to generate chimeric derivatives and single-site mutants derived from P450 2B1 and 2B2. The proteins used in these studies are depicted in Figure 2. The first of the two chimeric derivatives (KE) was based primarily on the 2B2 sequence and contained 5 amino acids derived from 2B1. The second chimeric derivative (BK) was based on the 2B1 sequence with five amino acids derived from 2B2. These five amino acids include those at positions 473 and 478 which are part of the sixth substrate recognition site (SRS) that was proposed by Gotoh (*13*) to be an important region in influencing substrate interactions with P450.

We have recently demonstrated a significant role for the Gly-478 residue in determining the catalytic activity of 2B1 and the susceptibility of the enzyme towards inactivation by benzyl aminobenzotriazole.[2] Similarly, Halpert and coworkers (*14*) have demonstrated that the Gly-478 of 2B1 has a significant influence on the catalytic activity and substrate specificity of the enzyme. Moreover, the various mutated P450s, whose Gly-478 was substituted by residues with larger side chains such as Leu, Thr, Ser, Val, and Ile, displayed different susceptibilities toward inactivation by chloramphenicol (*14*). In the present study, three single-site mutants have been generated based on the 2B2 sequence. The first mutant, 2B2-A478G, consisted of the 2B2 sequence with Ala-478 replaced by Gly, the corresponding residue in 2B1. The second mutant 2B2-G303S consisted of the 2B2 sequence with Gly-303 replaced by Ser, the amino acid in 2B1. This mutant was chosen since it falls within one of the substrate recognition regions of the P450 2 family and is immediately adjacent to the Thr residue that is highly conserved among all P450s. The third mutant was 2B2-A363V in which Ala-363 of 2B2 was mutated to Val. Ala-363 of 2B2 is located in the fifth SRS proposed by Gotoh (*13*). Moreover, mutation of the corresponding amino acid in 2B1 (from Val to Ala) influenced both the activity and regioselectivity for the oxidation of androstenedione and testosterone (*15*). Finally, the BK-A478G modified protein is identical to the BK chimeric-protein except for the inclusion of the additional A478G modification. These proteins, 2B1, 2B2, 3 chimeras, and 3 single amino acid mutants were used to study the effect of the protein sequence modifications on interactions with LIDO.

EXPERIMENTAL PROCEDURES

Materials. The cDNA clones for rat liver 2B1 and 2B2 were obtained from Dr. F. J. Gonzalez (National Cancer Institute, Bethesda, MD) and modified for expression in *E. coli* using the pCW *Ori*$^+$ expression vector as described (*16*). Primers for site-directed mutagenesis were obtained from the University of Michigan Macromolecular Core Facility (Ann Arbor, MI). Expression media for *E. coli* cultures was obtained from Difco (Detroit, MI). Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA) and Promega Corporation (Madison, WI). LIDO, δ-ALA, procaine, DLPC, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). IPTG was purchased from Alexis Biochemicals (San Diego, CA).

Construction and Expression of P4502B Chimeric Proteins. The two chimeric derivatives (BK and KE) were constructed using the internal *Bam*HI and *Kpn*I restriction enzyme sites at nucleotide positions 496 and 1141 with respect to the start codon of both cDNAs. A cloning-plasmid derived *Eco*RI site found 50 bp following the stop codon of either cDNA was used in the construction of the KE chimera. The 550 bp *Bam*HI−*Kpn*I or the 500 bp *Kpn*I−*Eco*RI DNA fragments derived from the 2B1 cDNA were purified

[2] Kent, U. M., Hanna, I. H., Szklarz, G. D., Vaz, A. D. N., Halpert, J. R., and Hollenberg, P. F. (1997) *Biochemistry 36*, 11707−11716.

following electrophoresis on a 0.8% agarose gel. The purified restriction fragments were ligated to the pCW2B2 expression plasmids devoid of the corresponding 2B2-derived restriction fragments. The sequences of the two resulting chimeras (BK and KE) were confirmed by DNA sequencing.

Site-Directed Mutagenesis. Mutagenesis of 2B2 residues Ala-478 to Gly, Gly-303 to Ser, and Ala-363 to Val were accomplished by PCR using the method described by Higuchi (*17*) using the following primers: (5′-GAGG-TATTTTTCCAATGCCACT-3′), (5′-GGTGCTGCTGGTCT-CAGTGCC-3′), and (5′ GCAGATCTTGTCCCAATTG-3′), respectively. The PCR products were sequenced at The University of Michigan DNA-sequencing facility to ensure the absence of any errors in the sequence that may have occurred following PCR-amplification. The purified PCR products were then digested with either *Bam*HI/*Kpn*I or *Kpn*I/*Eco*RI restriction enzyme-combinations and ligated into the P450 2B2 expression-plasmid that had been digested with the same endonuclease combinations, and purified as described above.

Expression and Purification of Mutated P450s. The various mutated derivatives were expressed in *E. coli* and subsequently purified using the media supplements, growth conditions, and purification procedures as described (*18*, *19*).

Spectral Binding Studies. Binding spectra were recorded on an Aminco DW2 spectrophotometer equipped with the OLIS operating system (On-Line Instruments Bogart, GA) essentially as described (*20*). The various P450s were suspended to a final concentration of 0.5−1.0 nmol/mL in a 50 mM Tris-HCl buffer, pH 7.4, containing 20% glycerol and 150 mM KCl and placed into two cuvettes (sample and reference). LIDO was added sequentially to the sample cuvette either in a methanolic solution or dissolved in water,[3] while the reference cuvette received an equal volume of the vehicle solvent. Difference spectra were recorded from 360 to 450 nm after each addition. Dissociation constants were determined using nonlinear fits of the average absorbance differences between 388 and 420 nm at various concentrations of LIDO.

The fraction of P450 in the high-spin form following LIDO addition was estimated from the second derivative analysis of the P450 absorption spectra in the absence or presence of 10 mM LIDO as described (*21*).

LIDO Oxidation Assays. Assays were conducted in 1.0 mL reaction volumes at 37 °C in 50 mM HEPES buffer, pH 7.4, containing 10 mM $MgCl_2$ and 0.1 mM EDTA. The P450 used in each reaction varied from 300 pmol for 2B2 to 50 pmol for 2B1. A reductase:P450 ratio of 2:1 was maintained in all cases. The P450s were mixed with reductase and freshly sonicated DLPC (10 μg/reaction) and placed on ice 1−2 h. Cytochrome b_5, when used, was added from a concentrated stock as part of the initial reconstitution mixtures at a ratio of 2:1 relative to the P450. Aliquots of the reconstitution mixtures were preincubated at 37 °C for 3 min in 990 μL of the reaction buffer containing the concentrations of LIDO indicated. NADPH (10 μL) was then added to a final concentration of 1.0 mM, and the reactions were terminated after 10 min by addition of 1.0 N NaOH (100 μL). Procaine (20 nmol) was added to all samples as an internal standard prior to extraction with 3.0 mL of ethyl acetate. Samples were then centrifuged at 500*g* in a bench-top centrifuge to separate the phases. The ethyl acetate layer was collected and evaporated under a stream of nitrogen. The dried samples were reconstituted with 200−600 μL of HPLC buffer A [0.1 M potassium phosphate, pH 3.0:acetonitrile (95:5) (v/v)]. Fifty microliter aliquots of the reconstituted samples were analyzed using a Gilson HPLC (Middleton, MA) equipped with a Rainin C-18 column, Gilson autosampler, UV detector set at 214 nm and a Hewlett-Packard integrator. The chromatography was carried out at a flow rate of 1.8 mL/min using a 95% buffer A and 5% buffer B [0.1 mM potassium phosphate buffer, pH 3.0:acetonitrile (1:1) (v/v)] mixture which was changed to a 50% buffer A and 50% buffer B mixture after 15 min. Quantitation of the metabolites formed was achieved by running standard curves generated using authentic procaine and MEGX standards.

Purification of Related Proteins. NADPH-cytochrome P450 reductase was expressed in *E. coli* and purified as described (*22*). Rat liver b_5 was purified as described (*11*).

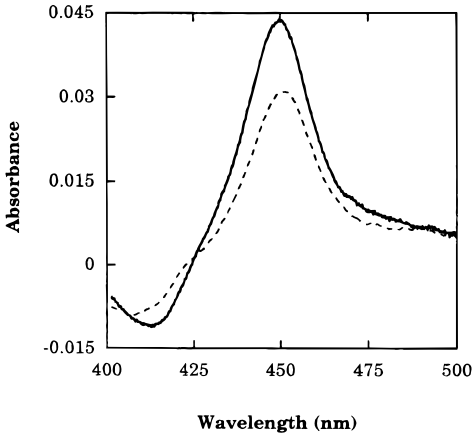

FIGURE 3: Reduced-CO difference spectra of the purified 2B chimeric proteins. The maxima were 450 nm and 451 nm for BK (—) and KE (- - -), respectively.

RESULTS

Expression and Purification of Modified Enzymes. The enzymes used for these studies are represented in Figure 2. All enzymes were expressed in *E. coli* and partially purified as described (*18*, *19*). The final specific content ranged from 3 to 8 nmol of P450/mg of protein. The partially purified recombinant proteins displayed reduced-CO spectra similar to the spectra observed with the purified rat liver 2B1 and 2B2 enzymes. Figure 3 shows the reduced-CO difference spectra observed for the two chimeric proteins demonstrating the small differences in the position of the maximum absorption (450 for BK and 451 for KE) that were similar to the results obtained previously with 2B1 and 2B2, respectively (*9*).

Determination of the Spectral Binding Constants for LIDO. The results of the binding experiments shown in Figure 4 and Table 1 demonstrate distinct differences in the way LIDO interacts with the different P450s. The addition of LIDO to 2B2, 2B2-G303S, 2B2-A478G, or the KE chimeric protein

[3] Initial experiments were performed with LIDO free base which was soluble in aqueous solutions up to 5.0 mM. In later experiments requiring the use of concentrations as high as 32 mM, we used the chloride salt of LIDO.

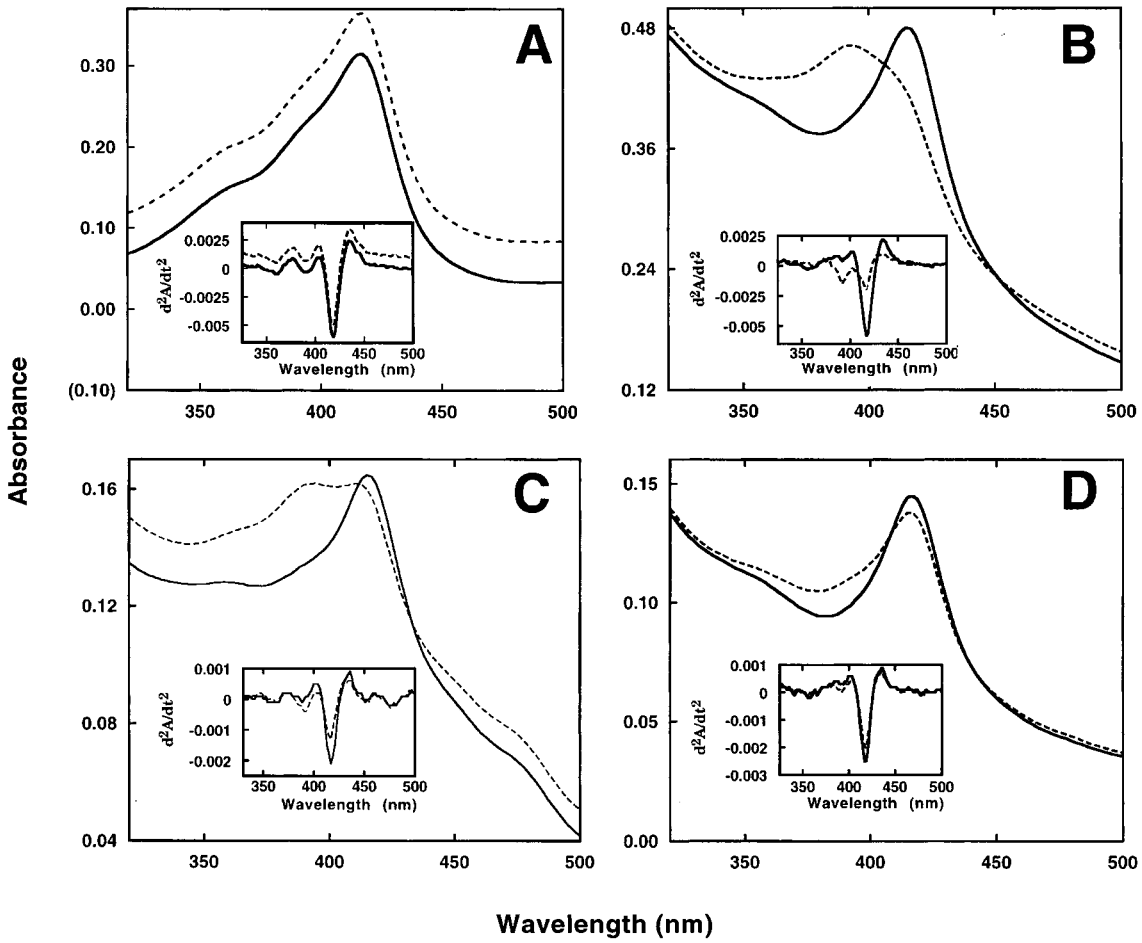

FIGURE 4: Effect of LIDO on the absorption spectra of 2B1 (panel A), 2B2 (panel B), 2B2-A478G (panel C), and 2B2-A363V (panel D). The spectra were determined as described in Experimental Procedures. The cuvette contained 0.5 nmol of P450 suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM KCl and 20% glycerol. The scans represent the absorption spectra of the proteins in the absence of LIDO (—) and in the presence of 10 mM LIDO(···). Scans shown in panel A were shifted by 0.05 absorbance units for clarity. The insets in each of the panels represent the second derivatives of the absolute spectra of the proteins in the presence of 10 mM LIDO (···) in the absence of LIDO (—).

Table 1: Lidocaine Binding to the Various P450s: Determination of the K_s Value and the High-Spin Iron Content of the Various P450s in the Absence or Presence of Lidocaine

P450	K_s[a]	% of Fe^{III} in high-spin form (−LIDO)[b]	% of Fe^{III} in high-spin form (+LIDO)[b]
2B2	19 ± 3.2	17	46
2B2-G303S	71 ± 6.7	20	67
2B2-A478G	48 ± 13	25	39
2B2-A363V	1120 ± 141	15	22
KE chimera	87 ± 2.1	28	47
BK chimera	5790 ± 140	19	19
BK-A478G	>7000	8.5	13
2B1	1652 ± 147	22	22

[a] Values represent means of duplicate experiments that were conducted as described in the Experimental Procedures. [b] The percentage of iron in the high-spin form in the presence or absence of 10 mM LIDO was estimated using the second derivative analysis of the P450 absorption spectra as described (21).

solutions evoked a typical type I binding spectrum (*20*). Such difference spectra are characterized by an increased absorbance at 388 nm that is accompanied by a decreased absorbance around 420 nm and are indicative of a shift in the spin equilibrium of the heme iron of the P450 (*23*). The changes in absorbance observed with these proteins were saturable with increasing amounts of LIDO. The differences in the absorbance between 388 and 420 nm and the corresponding LIDO concentrations were used to estimate the K_S values (*20*). With these four proteins, K_S values of less than 100 μM were obtained (Table 1). Under the same experimental conditions, the addition of LIDO to 2B2-A363V or the BK chimeric protein evoked similar type I binding spectra, but at much higher LIDO concentrations and with a significantly smaller change in the spin state of the heme iron (Table 1). The addition of LIDO to 2B1 and BK-A478G resulted in difference spectra that were distinctly different from those observed with the other proteins. Upon addition of LIDO (0−32 mM) to either 2B1 or BK-A478G, two peaks were observed in the difference spectra at 393 and 415 nm, while the trough was shifted to 425 nm (data not shown). Nonetheless, the magnitude of the difference in absorption between 393 and 415 were used to estimate the LIDO K_S values for both 2B1 and the BK-A478G protein that are reported in Table 1.

Estimation of the High-Spin Fraction of the P450 in the Presence of LIDO. The fractions of the different expressed P450s that were shifted to the high-spin configuration shown in Table 1 were estimated from the second derivative analysis of the absorption spectra in the absence or presence of LIDO (*21*). The proteins may be classified into three groups based on the fraction of the protein that was converted to the high-

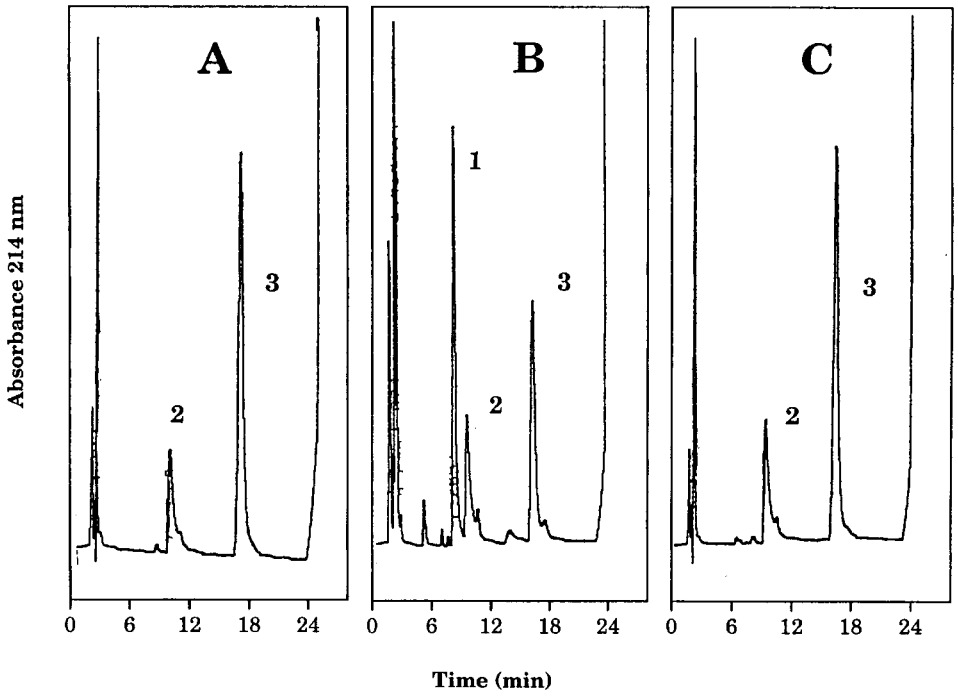

FIGURE 5: HPLC elution profiles for the metabolites of LIDO. The experimental conditions and chromatographic analysis were carried out as described in Experimental Procedures. Peaks 1−3 represent Me-OH-LID, procaine (internal standard), and MEGX, respectively. The reaction mixtures contained 50 pmol 2B1 (panel A), 300 pmol 2B2 (panel B), and 100 pmol 2B2-A363V (panel C).

Table 2: LIDO Oxidation by the Various Recombinant P450s: Determination of the Michaelis Constants for the Recombinant P450s in the Absence of b_5[a]

	MEGX			Me-OH-LID		
P450	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
2B2	61.6 ± 21.6	1.15 ± 0.26	18.7	39.1 ± 8.6	0.60 ± 0.080	15.3
2B2-G303S	181.8 ± 10.4	4.42 ± 3.6	24.3	95.2 ± 5.3	1.46 ± 1.3	15.3
2B2-A478G	34.00 ± 3.80	6.35 ± 0.45	187	21.1 ± 3.7	2.63 ± 0.19	125
2B2-A363V	1193 ± 259	60.0 ± 13	50.3	-[b]	-	
KE chimera	51.4 ± 1.8	11.9 ± 0.49	232	48.4 ± 0.50	2.83 ± 0.15	58.5
BK chimera	945 ± 271	28.8 ± 12	30.5	-[b]	-	
BK-A478G	2102 ± 158	222.4 ± 39	106	-[b]	-	
2B1	620.6 ± 49.0	213 ± 70	343	-[b]	-	

[a] Data represents average ± standard deviations of two experiments done in duplicate. Reactions were conducted as described in the Experimental Procedures. [b] No Me-OH-LID was detected.

spin configuration in the presence of saturating LIDO concentrations. The groups are 2B2 and 2B2-G303S (~30%); 2B2-A478G and the KE chimera (~15%); and 2B2-A363V, the BK chimera, BK-A478G, and 2B1, which exhibited less than 10% conversion to the high-spin configuration in the presence of LIDO.

Regioselectivity of LIDO Oxidation by the Recombinant P450s. The metabolites of LIDO can be easily resolved by HPLC (Figure 5). Under typical assay conditions, the GX metabolite, which results from the deethylation of MEGX (Figure 1), was undetectable. The HPLC profile for these products formed by oxidation by 2B1 shown in Figure 5A indicated formation of only MEGX, while both MEGX and Me-OH-LID were formed by 2B2 (Figure 5B). The mutation of Ala-363 in 2B2 to Val resulted in the complete loss of the Me-OH-LID metabolite (Figure 5C). Similar results were obtained following HPLC resolution of the LIDO metabolites formed by the other recombinant proteins. The BK chimera and BK-A478G proteins also formed MEGX exclusively. However, both metabolites were observed with the KE chimeric protein and the 2B2-G303S and 2B2-A478G mutants (data not shown).

Determination of Michaelis Constants for Oxidation of LIDO by the Recombinant P450s. The K_S values calculated from the spectral binding experiments were used to guide the selection of the appropriate concentration ranges for the determination of the Michaelis values. The Michaelis values were determined using nonlinear regression analysis of the rate of product(s) formed and the LIDO concentrations used. As shown in Table 2, the K_m values obtained for 2B2-G303S or 2B2-A478G were not vastly different than the K_m values for 2B2. The K_m values obtained with the KE chimera were also similar to those obtained with 2B2. However, the rates for the oxidation of LIDO by these modified proteins were 4−10-fold greater than the rate exhibited by 2B2. The enhanced rates were observed for the production of both MEGX and Me-OH-LID. Additionally, the K_m values calculated for these enzymes were in close agreement with the K_S values determined in the spectral binding experiments.

With 2B1, the BK-chimera, BK-A478G, and 2B2-A363V, the K_m values were much higher than those for 2B2. Similarly, the rates of LIDO oxidation by these enzymes were

Table 3: LIDO Oxidation by the Various Recombinant P450s: Determination of the Michaelis Constants for the Recombinant P450s in the Presence of Cytochrome b_5[a]

	MEGX			Me-OH-LID		
P450	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
2B2	85.50 ± 7.64	3.90 ± 0.28	45.6	33.5 ± 1.7	1.57 ± 0.04	46.9
2B2-G303S	100.4 ± 13.4	13.8 ± 2.10	138	86.6 ± 1.6	3.86 ± 0.62	44.6
2B2-A478G	37.70 ± 7.40	5.47 ± 0.11	145	19.7 ± 3.7	2.36 ± 0.05	120
2B2-A363V	972.0 ± 137	107 ± 11.3	110	-[b]	-	
KE chimera	45.60 ± 12.0	18.6 ± 1.30	408	48.6 ± 2.9	4.37 ± 0.34	89.9
BK chimera	583.0 ± 135	39.8 ± 24.2	68.3	-[b]	-	
BK-A478G	1837 ± 372	140 ± 28.6	76.2	-[b]	-	
2B1	505.5 ± 59.0	94.9 ± 5.00	188	-[b]	-	

[a] Data represent means ± standard deviations of two experiments done in duplicate. Reactions were conducted as described in Table 1. [b] No Me-OH-LID was detected.

25−200-fold higher than those observed with 2B2. The K_m value for 2B2-A363V was in close agreement with the K_S value from spectral binding experiments. Interestingly, the rate of oxidation exhibited by the 2B2-A363V mutant was approximately 2-fold greater than that exhibited by the BK chimera. However, upon incorporating the A478G modification into the BK chimera (to generate the BK-A478G protein), the rates exhibited by this protein were comparable to those rates exhibited by 2B1.

Effect of b_5 on LIDO Oxidation by the Recombinant P450s. The inclusion of b_5 in the initial reconstitution mixtures had different effects on the rates of LIDO oxidation depending on the identity of the P450s. Comparison of the values shown in Table 3 to those in Table 2 shows that no major differences were observed on the K_m values due to the inclusion of b_5. However, the rates of oxidation were influenced in a more significant manner. The inclusion of b_5 either enhanced (2B2, 2B2-G303S, and 2B2-A363V), had no significant effect (2B2-A478G, KE chimera, or BK chimera), or had an inhibitory effect (2B1 and the BK-A478G protein) on the rate of LIDO oxidation.

DISCUSSION

The oxidation of LIDO by the closely related P450s 2B1 and 2B2 was investigated in detail. The oxidation of LIDO by 2B1 proceeded at relatively high rates (greater than 150 nmol of product formed/nmol of P450/min), resulting exclusively in MEGX formation. P450 2B2 metabolized LIDO at much slower rates (less than 5 nmol/nmol of P450/min) leading to the generation of both MEGX and Me-OH-LID. The production of Me-OH-LID by 2B2 but not 2B1 suggested a possible difference in the orientation of LIDO in the active sites of the two enzymes. Therefore, we conducted spectral binding studies as a preliminary test of this hypothesis. The results of these studies indicated distinct differences in the interactions of LIDO with the two enzymes in their native (ferric) state. The interaction of LIDO with 2B2 resulted in a typical type I binding spectrum, indicating a substrate-induced change in the spin state of the heme iron from low spin to high spin (*23*). The value obtained for the maximal change in the absorbance was indicative of approximately 30% of the P450 undergoing a shift in the spin equilibrium to the high-spin form. The measured K_s value of approximately 20 μM indicated a relatively high affinity of 2B2 for LIDO. The difference spectrum resulting from the addition of LIDO to 2B1 was distinctly different from a typical type I binding spectra. Moreover, the addition of high concentrations of LIDO only resulted in small perturbations of the 2B1 spectrum, indicating that only a very small population of the enzyme (less than 1%) was shifted to the high-spin form.

Much of our understanding of substrate-induced shifts in the heme spin state equilibrium is based upon studies performed with P450 101 (*24*). For P450 101, the changes observed in the spectra of the protein due to substrate binding were further explained from studies on the crystal structures of P450 101 with substrates bound in its active site (*25*). The binding of substrates such as camphor and adamantane to P450 101 results in the displacement of the water molecule (or alteration in the distance of the iron-water ligand) that usually occupies the sixth coordination position of the heme iron (*26*). This step serves to shift the heme iron from the ferric low-spin to the high-spin form. An additional important aspect that emerged from these studies deals with those substrates that do not induce a complete shift in the heme equilibrium state. With these substrates, such as norcamphor, the bound substrate displayed a higher degree of movement within the active site. This indicates that such substrates are loosely bound and do not completely displace the iron-ligated water (or hydroxide ion).

The observed binding properties for LIDO and the products formed following LIDO oxidation by 2B1 and 2B2 suggest the possibility of two different binding orientations for LIDO within the active sites of 2B1 and 2B2. For 2B2, the aromatic portion of the molecule may be bound tightly in a position in close proximity to the activated oxidizing species. This orientation would permit both hydroxylation at the benzylic carbon and *N*-deethylation. Moreover, the presence of the aromatic portion of LIDO in close proximity to the heme would possibly be responsible for the observed high degree of shift of the heme spin state equilibrium. With 2B1, the aromatic portion of LIDO may either be a greater distance away from the oxidizing species and thus not able to greatly influence the spin state equilibrium or not tightly bound, and thus, it is not able to completely displace the water ligand in the sixth coordination position.

P450s can be contrasted with many dioxygenases in that they achieve catalysis by activating molecular oxygen as opposed to the organic substrate (*27*). Therefore, the oxidation of the substrates in this case would be a consequence of the interaction of the activated oxygen with the bound substrate. Moreover, the regioselectivity would be determined by the accessibility of target sites on the substrates and the relative reactivity of the various positions

on the substrate. With 2B1, the benzylic position of LIDO may not be accessible to the activated oxygen, and therefore, no Me-OH-LID is observed. With 2B2, the benzylic position may be accessible to the activated oxygen which leads to the formation of Me-OH-LID. The fact that 2B2 is also able to catalyze *N*-deethylation reactions even though the LIDO-nitrogen may be located further away from the activated oxygen, or because LIDO may not be tightly bound to 2B1 is not surprising in light of the results of studies on the chemistry of *N*-deethylation reactions. Studies of kinetic isotope effects (*28*) demonstrated that P450 mediated *N*-deethylations may be initiated by the donation of an electron from the nitrogen rather than the abstraction of a hydrogen from the adjacent carbon atom. Furthermore, studies with various substituted *N*,*N*-dimethylanilines demonstrated that electron transfers originating from the nitrogen atom may proceed over relatively far distances under proper conditions in the active site (*29*). Additional evidence to corroborate such a mechanism was observed in the *N*-dealkylation of deprenyl by P450 2D6 (*30*). Such an activity is atypical for 2D6 which usually catalyzes hydroxylation reactions that occur 5−7 Å away from the basic nitrogen. In this case, the basic nitrogen is believed to be held in place via electrostatic interactions with Asp-301 of 2D6 (*31*, *32*).

Upon comparing the rates of LIDO oxidation by the two enzymes, 2B2 had a much lower rate of catalysis than 2B1. In contrast, the K_m value of 2B2 for LIDO was approximately 10-fold lower than the K_m value of 2B1. In this special case, it appears that 2B1 is more suitable for maximization of the rate of LIDO oxidation than 2B2. On the basis of the discussions of enzyme−substrate complementarity effects on the rate of enzyme catalysis (*33*), the initial 2B2-LIDO complex may be more stable than the 2B1−LIDO complex. The higher stability of the initial P450−LIDO complex implies the need to expend more energy to proceed with later catalytic steps that involve disruption of the initial binding state. Consequently, the efficiency of the overall catalytic reaction as characterized by the k_{cat}/K_m value would be affected accordingly.

In order to determine which of the 13 amino acid differences between the two cytochromes are responsible for the differences in LIDO oxidation, a series of modified proteins derived from the two wild-type proteins was investigated. The recombinant proteins were classified into three groups based upon their spectral properties. The binding of LIDO to 2B2 and 2B2-G303S proteins led to a shift of approximately 30% of the P450 to the high-spin form. The fraction that underwent a shift to high spin was approximately 15% with 2B2-A478G and the KE chimera, whereas it was less than 10% with 2B2-A363V, the BK chimera, BK-A478G, and 2B1. Interestingly, those proteins that showed significant shifts in the spin equilibrium metabolized LIDO to produce both MEGX and Me-OH-LID. With the enzymes that exhibited less than 10% shift to the high-spin configuration, only MEGX was detected. This observation provided additional evidence that linked the orientation of binding of LIDO to the observed spectral shifts. The effect of the A363V mutation was to prevent the binding of LIDO in an orientation that would allow for the formation of Me-OH-LID. On the basis of this argument, the A363V mutation would appear to prevent the positioning of the aromatic portion of LIDO in the site that is accessible to the activated oxidizing species. The K_s measured with 2B2 was approximately 20 μM, indicating a relatively high affinity interaction. However, the K_s measured with the 2B2-A363V mutant was approximately 1.0 mM. With the elimination of that site, LIDO was no longer hydroxylated at the benzylic position and the rate of MEGX formation increased tremendously.

Another important aspect that is apparent from the oxidation data is the key role played by the residue at position 478 in three of the modified proteins. The A478G point mutant and the KE chimeric protein displayed approximately 4−10-fold higher catalytic efficiencies (k_{cat}/K_m) for LIDO oxidation than 2B2. Such an increase in the catalytic efficiency was observed for the generation of both Me-OH-LID and MEGX. The incorporation of the A478G point mutation into the BK chimeric protein to generate BK-A478G resulted in an almost 8-fold increase in the k_{cat} over that of BK chimera. However, the more than 2-fold increase in the K_m value over that for the BK-chimera resulted in an overall 3-fold increase in catalytic efficiency. Similar behavior was observed with the 2B1 mutant protein where Gly-478 was replaced by Ala[2] (*14*). This mutation resulted in catalytic activities that were much lower than the wild type 2B1.

The inclusion of b_5 in the initial reconstitution mixture had differential effects on LIDO oxidation by the various site-specific mutants. With 2B2, 2B2-G303S, 2B2-A363V, and the BK chimeric protein the catalytic activities (characterized by the formation of MEGX and Me-OH-LID) were increased by 1.5−3-fold. No significant effects were seen with 2B2-A478G and the KE chimera (approximately 1.5-fold or less), whereas significant inhibitory effects (30−50% inhibition) were observed on the activities of 2B1 and BK-A478G protein. Stoichiometry determinations for LIDO oxidation by 2B1 demonstrated a high degree of coupling with almost 68% of the consumed NADPH being coupled to the formation of MEGX. Alternatively, only about 20% of the NADPH consumed went into generating MEGX and Me-OH-LID during the oxidation of LIDO by 2B2. In the presence of b_5 the percentages of coupling increased to approximately 88 and 29% for 2B1 and 2B2, respectively.[4] The results of cytochrome b_5 effects on the catalytic activities of 2B1 and 2B2 are in support of the argument that the effect exerted by b_5 depends of the initial coupling state of the P450 during the oxidation of a certain substrate (*34*). The efficiently coupled LIDO oxidation by 2B1 would be inhibited due to b_5 whereas the poorly coupled LIDO oxidation by 2B2 would be stimulated.

The results of these studies have shown that although 2B2 binds LIDO with high affinity and exhibits a low K_m value during its metabolism the catalytic efficiency is rather poor. Alternatively, 2B1 exhibits a higher K_m value for LIDO however the oxidation proceeds with much higher efficiency. In these regards, 2B1 may be considered as an enzyme which evolved to be more suited for maximization of the rate of LIDO-oxidation over 2B2. The results of these studies also implicate Ala-363 and Ala-478 as important residues that participate in the active site of 2B2. The use of LIDO as an active site probe adds another advantage because of its

[4] Hanna, I. H., Hollenberg, P. F. Unpublished results.

observed effect on the spin state of the heme iron. Our results implicate the aromatic portion of the molecule as being responsible for affecting the spin-state equilibrium of the P450.

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