Site-Directed Mutageneses of Rat Liver Cytochrome P-450$_d$: Catalytic Activities toward Benzphetamine and 7-Ethoxycoumarin†

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ABSTRACT: Catalytic activities toward benzphetamine and 7-ethoxycoumarin of 11 distal mutants, 9 proximal mutants, and 3 aromatic mutants of rat liver cytochrome P-450$_d$ were studied. A distal mutant Thr319Ala was not catalytically active toward benzphetamine, while this mutant retained activity toward 7-ethoxycoumarin. Distal mutants Gly316Glu, Thr319Ala, and Thr322Ala displayed higher activities (k_{cat}/K_m) toward 7-ethoxycoumarin that were 2.4–4.7-fold higher than that of the wild-type enzyme. Although k_{cat}/K_m values of four multiple distal mutants toward benzphetamine were less than half that of the wild type, activities of these mutants toward 7-ethoxycoumarin were almost the same as or higher than the wild-type activity toward this substrate. The distal double mutant Glu318Asp, Phe325Tyr showed 6-fold higher activity than the wild-type P-450$_d$ toward 7-ethoxycoumarin. Activities of the proximal mutants Lys453Glu and Arg455Gly toward both substrates were much lower (less than one-seventh) than the corresponding wild-type activities. Catalytic activities of three aromatic mutants, Phe425Leu, Pro427Leu, and Phe430Leu, toward benzphetamine were less than 7% of that of the wild type, while the activities of these aromatic mutants toward 7-ethoxycoumarin were more than 2.5 times higher than the wild-type activity toward this substrate. From these findings, in conjunction with a molecular model for P-450$_d$, we suggest that (1) the relative importance to catalysis of various distal helix amino acids differs depending on the substrate and that these differences are associated with the size, shape, and flexibility of the substrate and (2) the proximal residue Lys453 appears to play a critical role in the catalytic activity of P-450$_d$, perhaps by participating in forming an intermolecular electron-transfer complex.

A family of protoheme-containing enzymes, collectively called cytochrome P-450 (P-450),[1] are involved in a variety of oxidative reactions (Sato & Omura, 1978; Ortiz de Montellano, 1986). Eukaryotic P-450 is a membrane-bound enzyme, and thus its tertiary structure may differ somewhat from the crystal structure of the soluble bacterial P-450$_{cam}$ (Poulos et al., 1985, 1986). In particular, microsomal membrane-bound P-450s are expected to contain two amino-terminal membrane-spanning helices that serve to anchor these enzymes in the endoplasmic reticulum membrane (Nelson & Strobel, 1988). Nevertheless, beyond this membrane-bound amino-terminal region, eukaryotic microsomal P-450s are probably similar enough to bacterial P-450$_{cam}$ to warrant limited structural comparison between corresponding residues, and alignments are available of more than 34 P-450 sequences from one bacterial and various eukaryotic species (Gotoh & Fujii-Kuriyama, 1989; Nelson & Strobel, 1988, 1989). We have used genetic engineering techniques (Blow et al., 1986), together with a three-dimensional model based on the crystal structure of P-450$_{cam}$ (Poulos et al., 1985), in an attempt to correlate structure with function in rat liver P-450$_d$.

We had previously developed an efficient expression system for rat liver P-450$_d$ (Ryan et al., 1980; Guengerich et al., 1982) in the yeast *Saccharomyces cerevisiae* (Shimizu et al., 1986). With oligonucleotide-directed mutagenesis, we have been able to test directly the effects of mutation of specific amino acids on catalysis by P-450$_d$. As a result of this previous study, we suggested that the proximal heme environment of membrane-bound P-450$_d$ may be similar to that of water-soluble P-450$_{cam}$ (Shimizu et al., 1988; Poulos et al., 1985, 1986).

In the present paper, we describe the effects on catalytic activity of P-450$_d$ resulting both from these previous proximal mutations and from mutation of distal and aromatic amino acids of P-450$_d$. On the basis of the kinetic values obtained, and of our molecular model of P-450$_d$, we discuss structure–function correlations in rat liver P-450$_d$.

Strategy

Distal Mutants. There are many species of eukaryotic P-450s. Although these enzymes have similar spectra and

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[1] Abbreviations: P-450, cytochrome P-450; P-450$_d$, cytochrome P-450$_d$; P-450$_{cam}$, cytochrome P-450 purified from *Pseudomonas putida* grown in the presence of camphor; benzphetamine, *N*,α-dimethyl-*N*-(phenylmethyl)benzeneethanamine; 7-ethoxycoumarin, 7-ethoxy-2*H*-1-benzopyran-2-one; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; 17β-estradiol, estra-1,3,5(10)-triene-3,17β-diol; D-camphor, (+)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one; metyrapone, 2-methyl-1,2-di-3-dipyridyl-1-propanone; phenobarbital, 5-ethyl-5-phenyl-2,4,6-(1*H*,3*H*,5*H*)-pyrimidinetrione; 3-methylcholanthrene, 1,2-dihydro-3-methylbenz[*j*]aceanthrylene; acetanilide, *N*-phenylacetamide; zoxazolamine, 5-chloro-2-benzoxazolamine. Cytochrome P-450$_d$ corresponds to P-450IA2 according to the recently proposed nomenclature for the P-450 gene family by Nebert et al. (1987).

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N D I F G A G F E T319	V T T A I F W	rat P-450$_d$
N D I F G A G F D T	V T T A I T W	mouse P_3-450
F D L F G A G F D T	I T T A I S W	rat P-450$_c$
L D L F G A G F D T	V T T A I S W	mouse P_1-450
L S L F F A G T E T	S S T T L R Y	rat P-450$_b$
L S L F F A G T E T	G S T T L R Y	rat P-450$_e$
N D I F G A G F D T	I T T A L S W	rabbit P-450$_{LM4}$
L S L F F A G T E T	T S T T L R Y	rabbit P-450$_{LM2}$
W D V F S A G T D T	T S N T L K F	rabbit P-450$_{3b}$
T E M L A G G V N T	T S M T L Q W	bovine P-450$_{scc}$
G L L L V G G L D T	V V N F L S F	*P. putida* P-450$_{cam}$

FIGURE 1: Amino acid sequences of P-450s in the distal helix region (Poulos et al., 1985; Gotoh & Fujii-Kuriyama, 1989; Nelson & Strobel, 1988). The standard single-letter code is as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

molecular weight and are even immunologically cross-reactive, many have different substrate specificities; for example, some act on exogenously administered drugs and others on endogenous steroid hormones or prostaglandins (Ortiz de Montellano, 1986). It is assumed that these differences in substrate specificity may be correlated with differences in structure of various P-450 species. It seems reasonable to postulate that only amino acids of the distal substrate-binding region of the enzyme have direct effects on substrate specificity. Other regions of the enzymes may be expected to have indirect effects on active-site conformation or electrostatic properties. With this caveat in mind, we examined control of substrate specificity by distal region amino acids. Recent crystallographic studies of bacterial P-450$_{cam}$ revealed the identities of amino acids that interact with the substrate, D-camphor, and that are located on the distal side of the heme plane in the enzyme (Poulos et al., 1985, 1986). In this region (the sequence of which is shown in Figure 1) there are two invariant amino acids, Gly316 and Thr319 (numbered according to rat liver P-450$_d$), and three highly conserved amino acids, Phe313, Ala315, and Thr322 (Poulos et al., 1985; Gotoh & Fujii-Kuriyama, 1989; Nelson & Strobel, 1988). Since they are in the active-site region of P-450$_{cam}$, these residues may influence substrate specificity and may even have effects on the activation of molecular oxygen during catalysis (Poulos et al., 1985). Moreover, all these residues are located in the distal helix (helix I in P-450$_{cam}$), and there are compelling arguments supporting the idea that the distal helix is similarly positioned in all P-450s (Poulos et al., 1985, 1986; Gotoh & Fujii-Kuriyama, 1989; Nelson & Strobel, 1988). We have made mutations of these distal invariant and conserved amino acids in P-450$_d$ to better understand their roles in the function of eukaryotic P-450s (Figure 2).

In addition to exploring how individual amino acids of the distal region contribute to substrate specificity in P-450s, we were also curious to find out whether we could mimic the kinetic parameters of some of the other species of P-450 by making changes only in the distal helix region of P-450$_d$. Thus, multiple mutations were made among distal residues from Asn310 to Phe325 of rat P-450$_d$, changing these to the corresponding residues of rat P-450$_c$ (M1), rabbit P-450$_{LM4}$ (M2), mouse P_3-450 (M3), and mouse P_1-450 (M4) (Figure 2). Component single mutants (Glu318Asp and Phe325Thr) of the multiple mutant M3 were also made.

Proximal Mutants. The most highly conserved sequence among the P-450s is the region surrounding the proximal Cys

Distal Mutants

Mutant Phe313Tyr; Mutant Ala315Ser; Mutant Gly316Glu;

Mutant Thr319Ala; Mutant Thr322Ala;

Mutant M1: rat P-450$_d$ --- rat P-450$_c$

Asn310Phe, Ile312Leu, Glu318Asp, Val320Ile, Phe325Ser

Mutant M2: rat P-450$_d$ --- rabbit P-450$_{LM4}$

Glu318Asp, Val320Ile, Ile324Leu, Phe325Ser

Mutant M3: rat P-450$_d$ --- mouse P_3-450

Glu318Asp, Phe325Thr

Mutant Glu318Asp; Mutant Phe325Thr

Mutant M4: rat P-450$_d$ --- mouse P_1-450

Asn310Leu, Ile312Leu, Glu318Asp, Phe325Ser

Proximal Mutants

Mutant Gly450Ser; Mutant Leu451Ser; Mutant Lys453Glu;

Mutant Arg454Leu; Mutant Arg454His; Mutant Arg455Gly;

Mutant Ile457Ser; Mutant Glu459Ala; Mutant Ile460Ser;

Aromatic Mutants

Mutant Phe425Leu; Mutant Pro427Leu; Mutant Phe430Leu;

FIGURE 2: P-450$_d$ mutants studied in this work. Mutants were produced by site-directed mutagenesis as described under Experimental Procedures.

ligand. In the P-450$_{cam}$ structure certain key residues such as an invariant Phe contact the proximal heme surface and a His forms a hydrogen bond with a heme propionate. We therefore probed this region of P-450$_d$ using mutagenesis techniques to study the role of potentially important proximal side residues on catalysis.

Aromatic Mutants.[2] Gotoh and Fujii-Kuriyama (1989) suggested that there is a well-conserved aromatic region in animal P-450s. This region consists of three aromatic amino acids (A_i), two prolines (P), and either Arg or His (B) in the sequence A_1-X-X-P-X-X-A_2-X-P-X-B-A_3 (X represents weakly conserved amino acids). The corresponding sequence in rat liver P-450$_d$ is Trp-Lys420-Asp-Pro-Phe-Val-Phe425-Arg-Pro-Glu-Arg-Phe430. To better understand whether the amino acids in this region contribute to catalysis, we made three aromatic mutants, Phe425Leu, Pro427Leu, and Phe430Leu, and examined their effects on the catalytic activity of P-450$_d$.

Catalytic activities of the aromatic, proximal, and single and multiple distal mutants described above (see also Figure 2) were measured with benzphetamine and with 7-ethoxycoumarin as substrates. Using our molecular model of rat liver P-450$_d$, we were able to formulate limited structural explanations for these data.

Experimental Procedures

An expression vector consisting of P-450$_d$ cDNA and acid phosphatase promoter was constructed as described previously (Shimizu et al., 1986). Host cells, *S. cerevisiae* AH22 (*a leu2 his4 can1 cir*$^+$), were cultured as described previously (Shimizu et al., 1986, 1988).

Site-directed mutageneses for proximal mutants were done as previously described (Shimizu et al., 1988; Taylor et al., 1985a,b). For site-directed mutageneses of distal and aromatic

[2] Although Pro is not aromatic, we tentatively designated mutants of the "aromatic region" as "aromatic mutants" for convenience.

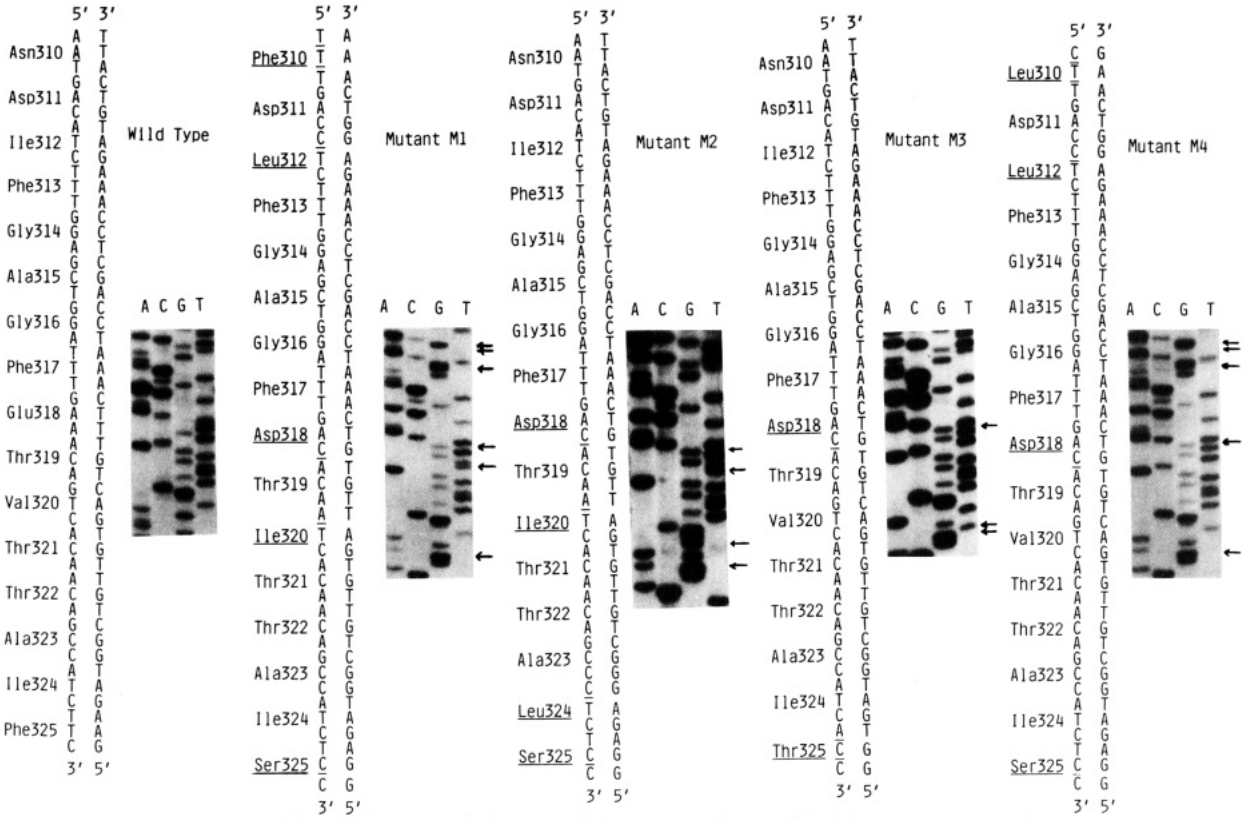

FIGURE 3: Nucleotide sequences of the wild-type and distal multiple mutants M1–4 of P-450$_d$.

mutants, we synthesized 15 oligonucleotides by Gene Assembler (Pharmacia) as follows:

(1) for single mutations

3'TTACTGTAGATACCTCGACCT5'(D8)(for Phe313Tyr)

3'GTAGAAACCTAGACCTAAACT5'(D9)(for Ala315Ser)

3'AAACCTCGACTTAAACTTTGT5'(D10)(for Gly316Glu)

3'ACCTAAACTTCGTCAGTGTTG5'(D11)(for Thr319Ala)

3'TTTGTCAGTGTCGTCGGTAGAAG5'(D12)(for Thr322Ala)

3'CCTAAACTGTGTCAGT5'(D15)(for Glu318Asp)

3'CGGTAGAGGACCTCGTA5'(D17)(for Phe325Ser)

3'GTTGTCGGTAGTGGACCTCGTA5'(D19)(for Phe325Thr)

3'GAAACACAATGCGGGTCT5'(D35)(for Phe425Leu)

3'CAAGGCGGATCTCGCC(D36)(for Pro427Leu)

3'CTCGCCAATGAATGGTT5'(D38)(for Phe430Leu)

(2) for double mutations

3'GTTGTAACAGAAACTGGAGAAACCT5'(D29)(for Asn310Phe, Ile312Leu)

3'CCTAAACTGTGTTAGTGTTGT5'(D30)(for Glu318Asp, Val320Ile)

3'GTTGTCGGGAGAGGACCTCG5'(D31)(for Ile324Leu, Phe325Ser)

3'CAGTTGTAACAGGAACTGGAGAAACCT5'(D32)(for Asn310Leu, Ile312Leu)

The underlined nucleotides designate the position of the mutations. Mutation was confirmed by determinations of nucleotide sequences by dideoxy chain terminater method using [α-^{32}P]deoxyCTP (Sanger et al., 1977). A sequencing primer used for the distal mutants was a 21-mer that corresponds to nucleotides from Leu345 to Arg351. A sequencing primer used for the aromatic mutants was a 21-mer that corresponds to nucleotides from Asn435 to Thr441. For multiple mutations such as mutants M1–4, we used stepwise the oligonucleotide after confirmation of the first mutation. Namely, for construction of mutant M1, oligomers D29, D30, and D17 were stepwise used. Likewise, for mutant M2, we used oligonucleotides D30 and D31; for mutant M3, we used oligonucleotides D15 and D19; and for mutant M4, we used oligonucleotides D32, D15, and D17. Figure 3 shows representative nucleotide sequences of the mutants M1–4 in this region. We strictly checked every mutant for whether a mutation(s) at another position(s) occurred or not.

Benzphetamine hydrochloride was a generous gift from Dr. T. Kamataki, Hokkaido University. NADPH was the product of Oriental Yeast Co., Tokyo. Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto), Toyobo (Osaka), Boehringer Mannheim (Mannheim, W. Germany), and Amersham International (Amersham, U.K.). Other reagents used were of the highest guaranteed grade and were used without further purification.

Yeast microsomes were prepared by crushing cells with the Bead-Beater (Biospec Products) or with MSK cell homogenizer (B. Braun, W. Germany). Microsomes were suspended in 100 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM DTT at the protein concentration of 2 mg/mL. NADPH–cytochrome P-450 reductase was purified as described previously (Taniguchi et al., 1979). P-450 concentration was spectrally determined from ferrous–CO complex vs ferrous difference spectra, with an $\epsilon_{447-490nm}$ value of 91 mM^{-1} cm^{-1} (Omura & Sato, 1964). Protein concentrations were determined by the Coomassie Brilliant Blue method (Bradford, 1976).

Benzphetamine N-demethylation activity was assayed in a 500-μL reaction mixture consisting of 0.1–1.5 nmol of P-450$_d$ in yeast microsomes, 400 units/nmol of P-450$_d$ NADPH–cytochrome P-450 reductase, 500 nmol of benzphetamine, and 0.1 M potassium phosphate buffer (pH 7.0). After the solution

was incubated at 37 °C for 5 min, the reaction was started by adding 500 nmol of NADPH. The reaction mixture was incubated for 20 min at 37 °C and then terminated by adding 250 μL of 10% trichloroacetic acid. The product was quantitated by monitoring formaldehyde formation (van der Hoeven et al., 1974).

7-Ethoxycoumarin *O*-dealkylase activity was assayed in a 500-μL reaction mixture consisting of 0.1–1.5 nmol of P-450$_d$ in yeast microsomes, 400 units/nmol P-450$_d$ NADPH–cytochrome P-450 reductase, 250 nmol of 7-ethoxycoumarin, and 0.1 M potassium phosphate buffer (pH 7.0). The reaction mixture was incubated for 20 min at 37 °C. The reaction was terminated by adding 100 μL of 2 N HCl. The turnover number was obtained by quantitation of 7-hydroxycoumarin fluorescence (Guengerich, 1978). Activities were determined under conditions in which the rate of product formation was constant with respect to protein and time of incubation.

Difference spectra were recorded with a Shimadzu UV spectrophotometer (UV-365) equipped with an end-on photomultiplier (Hamamatsu R-375). Fluorescence spectra were obtained with a Shimadzu spectrofluorophotometer (RF-500).

A molecular model of rat liver P-450$_d$ was generated by replacing amino acid side chains in the crystal structure of P-450$_{cam}$ (Poulos et al., 1985, 1986) with the corresponding side chains of P-450$_d$ according to the sequence alignment of Nelson and Strobel (1988), using the macromolecular modeling program FRODO (Jones, 1982) as implemented on an Evans and Sutherland PS390 graphics system. No attempts were made to model insertions in the P-450$_d$ sequence relative to the P-450$_{cam}$ sequence nor were "ends" joined in the P-450$_d$ model, generated due to deletions in the P-450$_d$ sequence, relative to P-450$_{cam}$.

Results

Distal Mutants. Steady-state kinetics and Lineweaver–Burk plots of benzphetamine demethylation were obtained for the wild type and for the distal mutants. Table I shows catalytic activities toward benzphetamine of the wild and mutant P-450$_d$s. Benzphetamine demethylation activity of the mutant Thr319Ala was undetectable. With the exception of the mutant Gly316Glu, most of the k_{cat} values of distal mutants were lower (1/2.6 to 1/7.9) than the corresponding wild-type value. Again with the exception of the mutant Gly316Glu, K_m values of all of the distal mutants were much lower (1/1.7 to 1/3.2) than that of the wild type. Catalytic activities (k_{cat}/K_m) of the distal mutants Ala315Ser, Gly316Glu, and Thr322Ala toward benzphetamine were similar to that of the wild type, while those of the distal mutants Phe313Tyr and multiple distal mutants M1–4 were much lower (1/1.9 to 1/4.3) than that of the wild type. Note that the apparent dissociation constant (approximately 0.1 mM) of benzphetamine from the mutant Thr319Ala, which was obtained from the Soret absorption spectral change, was similar to that (approximately 0.1 mM) of the wild type, suggesting that benzphetamine binds to this mutant in the same way as to the wild type.

Table II summarizes catalytic activities toward 7-ethoxycoumarin of the wild-type and mutant P-450$_d$s. All distal mutants had catalytic activity toward 7-ethoxycoumarin, in contrast to the observation that the mutant Thr319Ala had no activity toward benzphetamine. The k_{cat} value of the mutant Ala315Ser was half that of the wild type, while those of the mutants Phe313Tyr, Gly316Glu, Thr319Ala, and Thr322Ala and of the multiple mutant M3 (Figure 2) were higher (1.8–3.4-fold) than that of the wild type. The k_{cat} values of other distal mutants M1, M2, and M4 toward 7-ethoxycoumarin were all similar to the wild-type value. The K_m values of these three distal mutants toward 7-ethoxycoumarin were almost the same as or a little higher than that of the wild type, whereas that of the mutant M3 was considerably lower. Catalytic activities (k_{cat}/K_m) toward 7-ethoxycoumarin of the mutants Gly316Glu, Thr319Ala, and Thr322Ala and of M3 were much higher (2.4–6.1-fold) than that of the wild type; those of mutants Phe313Tyr and M1 were somewhat higher and those of mutants Ala315Ser, M2, and M4 were somewhat lower than the wild-type activity. It was especially interesting that the catalytic activity (k_{cat}/K_m) of the mutant M3 toward 7-ethoxycoumarin was greater than 6 times that of the wild-type enzyme.

Table I: Catalytic Activities toward Benzphetamine of Wild and Mutant P-450$_d$[a]

	k_{cat}	K_m	k_{cat}/K_m
rat liver P-450$_d$	3.9,[b] 11[c]		
expressed wild P-450$_d$	10.24 (2.00)[d]	0.240	42.67
pAM82[e]	<0.01		
distal mutants			
mutant Phe313Tyr	2.27	0.100	22.70
mutant Ala315Ser	3.85	0.074	52.03
mutant Gly316Glu	8.55	0.203	42.12
mutant Thr319Ala	<0.01		
mutant Thr322Ala	2.92	0.055	53.09
mutant M1, P-450$_d$–P-450$_c$	2.87	0.144	19.93
mutant M2, P-450$_d$–P-450$_{LM4}$	1.81	0.116	15.56
mutant M3, P-450$_d$–P$_3$-450	1.29	0.129	10.00
mutant Glu318Asp	1.15	0.144	7.99
mutant Phe325Thr	1.20	0.131	9.16
mutant M4, P-450$_d$–P$_1$-450	1.55	0.074	20.95
proximal mutants			
mutant Gly450Ser	<0.01		
mutant Leu451Ser	2.92	0.055	53.09
mutant Lys453Glu	<0.01		
mutant Arg454Leu	1.77	0.130	13.62
mutant Arg454His	1.86	0.238	7.82
mutant Arg455Gly	1.15	0.205	5.61
mutant Ile457Ser	1.85	0.141	13.12
mutant Glu459Ala	2.90	0.131	22.14
mutant Ile460Ser	1.81	0.116	15.61
aromatic mutants			
mutant Phe425Leu	0.56	0.048	11.67
mutant Pro427Leu	<0.01		
mutant Phe430Leu	0.72	0.091	7.91
rat liver P-450$_c$	6.7,[b] 13[c]		
rabbit liver P-450$_{LM4}$	6.4[f]		
mouse liver P$_3$-450			
mouse liver P$_1$-450			

[a] k_{cat}, turnover number expressed by min^{-1} obtained from V_{max}; K_m, mM; k_{cat}/K_m, mM^{-1} min^{-1}. Kinetic study for each mutant was repeated at least three times, and their averaged values are described. Experimental errors were less than 20%. [b] Ryan et al. (1980). [c] Guengerich et al. (1982). [d] This activity was obtained in terms of nmol min^{-1} (nmol of P-450$_d$)$^{-1}$ when benzphetamine N-demethylation activity was assayed by the methods of Ryan et al. (1980) and van der Hoeven et al. (1974). When assay conditions were changed and V_{max} values were obtained as described under Experimental Procedures, the apparent turnover number increased to 10.24 min^{-1}. [e] Microsomes of the yeast AH22 harboring a shuttle vector, pAM82, lacking P-450$_d$ cDNA. [f] Koop and Coon (1979).

Proximal Mutants. Catalytic activities of the proximal mutants Gly450Ser and Lys453Glu toward benzphetamine were not detected (Table I). The k_{cat} values of other proximal mutants toward benzphetamine were much lower (1/3.5 to 1/8.9) than that of the wild type. K_m values of the proximal mutants toward benzphetamine were also lower (1/1.7 to 1/4.4) than that of the wild type, with the exception of the mutants Arg454His and Arg455Gly, whose K_m values were

Table II: Catalytic Activities toward 7-Ethoxycoumarin of Wild and Mutant P-450$_d$[a]

	k_{cat}	K_m	k_{cat}/K_m
rat liver P-450$_d$	0.5,[b] 3.6[c]		
expressed wild P-450$_d$	0.21	0.084	2.50
pAM82[d]	<0.01		
distal mutants			
mutant Phe313Tyr	0.38	0.139	2.73
mutant Ala315Ser	0.11	0.071	1.55
mutant Gly316Glu	0.72	0.061	11.80
mutant Thr319Ala	0.71	0.100	7.10
mutant Thr322Ala	0.52	0.086	6.05
mutant M1, P-450$_d$–P-450$_c$	0.23	0.073	3.15
mutant M2, P-450$_d$–P-450$_{LM4}$	0.23	0.106	2.17
mutant M3, P-450$_d$–P$_3$-450	0.53	0.035	15.14
mutant Glu318Asp	0.30	0.064	4.69
mutant Phe325Thr	0.19	0.041	4.63
mutant M4, P-450$_d$–P$_1$-450	0.23	0.124	1.85
proximal mutants			
mutant Gly450Ser	0.31	0.060	5.17
mutant Leu451Ser	0.23	0.092	2.50
mutant Lys453Glu	<0.01		
mutant Arg454Leu	0.13	0.052	2.50
mutant Arg454His	0.39	0.060	6.50
mutant Arg455Gly	0.04	0.141	0.28
mutant Ile457Ser	<0.01		
mutant Glu459Ala	0.51	0.079	6.46
mutant Ile460Ser	0.19	0.145	1.31
aromatic mutants			
mutant Phe425Leu	0.18	0.028	6.43
mutant Pro427Leu	0.26	0.030	8.67
mutant Phe430Leu	0.26	0.040	6.50
rat liver P-450$_c$	37.3,[b] 27[c]		
rabbit liver P-450$_{LM4}$	0.02[e]		
mouse liver P$_3$-450	~1.5[f]		
mouse liver P$_1$-450			

[a] k_{cat}, turnover number expressed by min^{-1} obtained from V_{max}; K_m mM; k_{cat}/K_m, mM^{-1} min^{-1}. Kinetic study for each mutant was repeated at least three times, and their averaged values are described. Experimental errors were less than 20%. [b] Ryan et al. (1980). [c] Guengerich et al. (1982). [d] Microsomes of the yeast AH22 harboring a shuttle vector, pAM82, lacking P-450$_d$ cDNA. [e] Johnson and Muller-Eberhard (1977). [f] Ohyama et al. (1984). Activity was obtained for the major form of methylcholanthrene-induced mouse liver. Approximately 17% P$_1$-450 was mixed in this preparation.

comparable to the wild-type value. Catalytic activity (k_{cat}/K_m) of the mutant Leu451Ser toward benzphetamine was higher than that of the wild type, while those of other mutants were much lower (1/1.9 to 1/7.6) than that of the wild type. From the Soret spectral change, it was shown that benzphetamine binds to the mutants Gly450Ser and Lys453Glu with dissociation constants (approximately 0.1 mM) similar to that of the wild type.

The proximal mutants Lys453Glu and Ile457Ser had no catalytic activity toward 7-ethoxycoumarin (Table II). The k_{cat} value of the mutant Arg455Gly was much lower (1/5.3) than that of the wild type, while those of the mutants Arg454His and Glu459Ala were higher (1.9–2.4-fold) than that of the wild type. The k_{cat} values of other proximal mutants, Gly450Ser, Leu451Ser, Arg454Leu, and Ile460Ser, did not change so much from the wild-type value. K_m values of the proximal mutants Arg455Gly and Ile460Ser were higher than that of the wild type, while those of other mutants were more similar to that of the wild type. Catalytic activities (k_{cat}/K_m) of the proximal mutants Gly450Ser, Arg454His, and Glu459Ala toward 7-ethoxycoumarin were more than 2-fold higher than that of the wild type, while that of the mutant Arg455Gly was much lower (1/8.9) than that of the wild type. It was shown that 7-ethoxycoumarin binds to the mutants

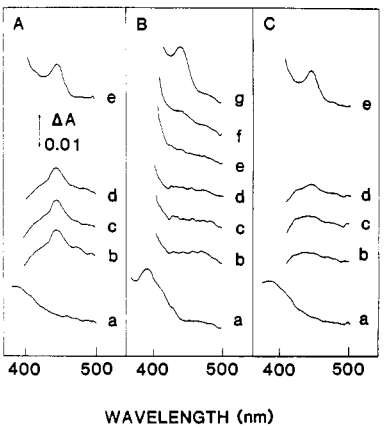

FIGURE 4: CO difference spectra of NADPH-P-450 reductase complexes with partially purified wild-type and mutant P-450$_d$s. (A) Solution consisted of (a) wild-type P-450$_d$ (0.13 μM) alone and (b–e) CO gas (approximately 0.5 mM), NADPH-P-450 reductase (150 units), and wild-type P-450$_d$ (0.13 μM). Spectrum a is absolute spectrum, while spectra b–d are difference spectra and were obtained at 0, 5, and 15 min after addition of 500 μM NADPH. Spectrum e was measured after addition of a trace of sodium dithionite to the solution (d). (B) Solution consisted of (a) mutant Lys453Glu (0.19 μM) alone and (b–f) CO gas (approximately 0.5 mM), NADPH-P-450 reductase (300 units), and mutant Lys453Glu (0.19 μM). Spectrum a is absolute spectrum, while spectra b–f are difference spectra and were obtained at 0, 5, 15, 25, and 40 min after addition of 500 μM NADPH. Spectrum g was obtained after addition of a trace of sodium dithionite to the solution (f). (C) Solution consisted of (a) mutant Arg455Gly (0.15 μM) alone and (b–d) CO gas (approximately 0.5 mM), NADPH-P-450 reductase (150 units), and mutant Arg455Gly (0.15 μM). Spectrum a is absolute spectrum, while spectra b–d are difference spectra and were obtained at 0, 5, and 15 min after addition of 500 μM NADPH. Spectrum e was obtained after addition of a trace of sodium dithionite. We obtained essentially the same results in the presence of the appropriate amount of dilauroyl-L-α-phosphatidylcholine and dimyristoyl-L-α-phosphatidylcholine (Müller-Enoch et al., 1984). Wild-type P-450$_d$ and mutants Lys453Glu and Arg455Gly were partially purified by ω-aminohexyl-Sepharose chromatography (unpublished experiments).

Lys453Glu and Ile457Ser with dissociation constants (approximately 0.1 mM) similar to that of the wild type.

CO Difference Spectra of the Mutants Lys453Glu- and Arg455Gly–NADPH–P-450 Reductase Complexes. The mutant Lys453Glu did not show any activity toward four substrates examined as described previously. To clarify whether or not the mutant Lys453Glu was reducible by the electron from NADPH through NADPH–P-450 reductase, we studied reducibility of the mutant by NADPH through NADPH–P-450 reductase in the presence of CO. As shown in Figure 4, the solution consisting of partially purified mutant Lys453Glu, NADPH–P-450 reductase, NADPH, and CO did not show a peak around 450 nm, which is characteristic of the reduced P-450–CO complex, while the solution containing partially purified wild-type P-450$_d$, NADPH–P-450 reductase, NADPH, and CO certainly showed a peak around 450 nm. Since reduction of the mutant Lys453Glu solution by sodium dithionite gave a characteristic CO difference spectrum around 450 nm, it is highly likely that an electron was not transferred from NADPH to the heme iron in this mutant through NADPH–P-450 reductase. Thus, it is suggested that Lys453 of P-450$_d$ is somehow involved in the interaction with NADPH–P-450 reductase (French et al., 1980; Müller-Enoch et al., 1984).

Since the mutant Arg455Gly had very low activities toward the two substrates examined, the electron transfer was also considered to be impeded in this mutant. As shown in Figure 4C, the reduction rate of the mutant Arg455Gly by NADPH through NADPH–P-450 reductase was much slower as com-

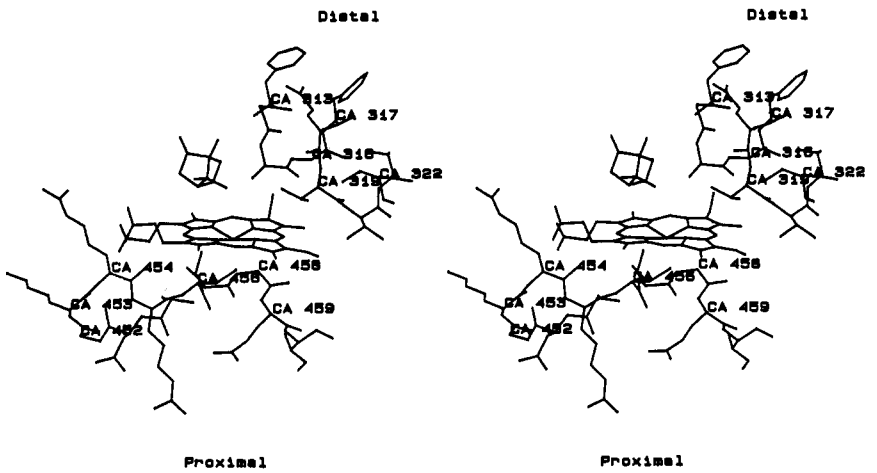

FIGURE 5: View of the proximal and distal regions in P-450$_d$. This hypothetical model was constructed by placing the P-450$_d$ sequence on the P-450$_{cam}$ backbone. The regions shown include the highly conserved Cys ligand peptide (proximal) and the distal helix. The sequence numbering is that of P-450$_d$ and shows those residues that were mutated. The invariant Cys ligand is at position 456. The location of the camphor molecule in P-450$_{cam}$ also is shown as a guide to the approximate location of substrates in P-450$_d$.

pared with that of the wild type. Significant reduction was, however, certainly observed for this mutant, in contrast with the mutant Lys453Glu. The reduction of the mutant Glu459Ala under the same conditions was as slow as that of the mutant Arg455Gly. All other distal, proximal, and aromatic mutants were reduced much faster than the three proximal mutants Lys453Glu, Arg455Gly, and Glu459Ala, and their reduction was completed within 20 s under the same conditions.

Aromatic Mutants.[2] The aromatic mutant Pro427Leu had no activity toward benzphetamine (Table I). The k_{cat} values of other aromatic mutants Phe425Leu and Phe430Leu toward benzphetamine were much lower (1/18.3 to 1/14.2) than that of the wild type. K_m values of the aromatic mutants Phe425Leu and Phe430Leu were lower (1/2.6 to 1/5) than that of the wild type. Catalytic activities (k_{cat}/K_m) of the aromatic mutants Phe425Leu and Phe430Leu were lower (1/3.7 to 1/5.4) than that of the wild type.

In contrast to the activity of the aromatic mutants toward benzphetamine, the three aromatic mutants had sufficient activities toward 7-ethoxycoumarin, and their k_{cat} values were almost the same as that of the wild type (Table II). K_m values of the three aromatic mutants were less than half that of the wild type. Catalytic activities (k_{cat}/K_m) of the aromatic mutants were higher (2.6–3.5-fold) than that of the wild type.

Discussion

Distal Mutants. In the region between Asn310 and Phe325, there are two amino acids, Gly316 and Thr319, that are invariant in 37 P-450 sequences (Gotoh & Fujii-Kuriyama, 1989; Nelson & Strobel, 1988). From the crystal structure of P-450$_{cam}$ (Poulos et al., 1985), it is known that the invariant Thr, corresponding in the sequence alignment of Gotoh and Fujii-Kuriyama (1989) to Thr319 of rat liver P-450$_d$, is located on the distal side of the heme plane (Figure 5), in a location where it would contact the substrate and/or molecular oxygen. Indeed, the crystal structure of the ternary camphor–CO–ferrous P-450$_{cam}$ complex shows that Thr319 does contact both the substrate and ligand as predicted (Raag and Poulos, unpublished results). In P-450$_{cam}$, the side-chain oxygen atom of this Thr introduces a distortion into the distal helix, widening it by hydrogen bonding to the carbonyl oxygen of the amino acid four residues downstream (Poulos et al., 1985). Thus, this helical distortion may be an important feature in catalysis by P-450s. As expected, the mutant Thr319Ala had no catalytic activity toward benzphetamine. However, this mutant

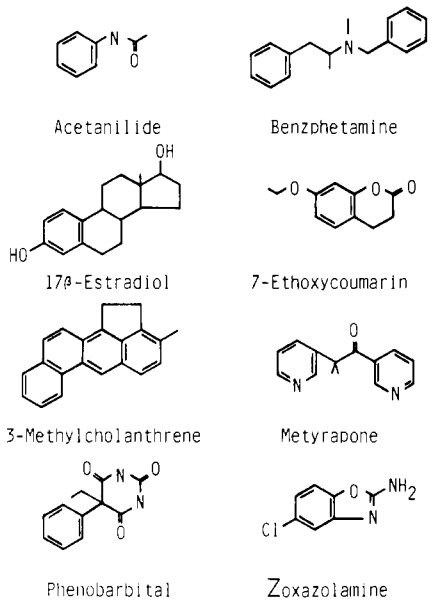

FIGURE 6: Structures of the P-450 substrates and inducers discussed in this work.

was active toward 7-ethoxycoumarin, having k_{cat} and k_{cat}/K_m values nearly 3 times those of the wild type. This mutant was also active against the substrates acetanilide (Furuya et al., 1989), zoxazolamine, and 17β-estradiol (our unpublished observations). We therefore conclude that Thr319 is necessary for activity toward some substrates, but not for others.

A possible explanation for differences in activity may rest with the structure of the substrates. As can be seen from Figure 6, benzphetamine consists basically of two phenyl rings separated by a chain of four atoms; torsional rotation is possible about each of these four atoms. In contrast, 7-ethoxycoumarin, zoxazolamine, and 17β-estradiol are essentially comprised of fused ring systems and hence are much more rigid molecules. Acetanilide also has fewer possible conformations than does benzphetamine. Replacing Thr319 with a helix-stabilizing residue, Ala, is likely to remove or decrease the distortion in the distal helix and thus force an O_2 ligand to adopt an alternate conformation. This new conformation may be incompatible with the correct orientation for benzphetamine but not with that of the smaller 7-ethoxycoumarin. An alternative but somewhat more speculative explanation is that the bump, which the threonine side chain forms as it protrudes into the active-site pocket, somehow enables, by reducing the confor-

mations they can adopt in the active site, substrates with large numbers of degrees of freedom, such as benzphetamine, to assume a proper orientation for subsequent catalysis. 7-Ethoxycoumarin, in contrast, is primarily a planar molecule and would not be expected to need a complementary enzyme surface against which to orient itself for catalysis. In such cases, the threonine side chain of the wild-type enzyme may hinder the movement and orientation of a rigid substrate, perhaps accounting for the enhanced activity of the Thr319Ala mutant against 7-ethoxycoumarin. The fact that threonine is so highly conserved in this position (Gotoh & Fujii-Kuriyama, 1989; Nelson & Strobel, 1988) and that P-450s are generally able to accommodate a variety of substrates may reflect a compromise between the need for a small Thr side chain to maintain a large open active site for rigid substrates with fused ring systems and the need for some projecting feature in the active site to facilitate the orientation of flexible substrates.

When the invariant Gly316 was changed to Glu, the kinetic parameters of benzphetamine metabolism by the mutant enzyme were not much different from those of the wild type toward this substrate, whereas catalysis of 7-ethoxycoumarin by the mutant was enhanced with respect to the wild type. In analogy with the P-450_{cam} structure, Gly316 is located in the distal helix, near one side of the heme surface (Figure 5). Again from our molecular model of P-450_d, the Glu316 side chain is expected to extend internally into the protein toward the location of a cluster of internal water molecules in P-450_{cam}, which it could potentially replace to interact with another highly conserved residue, Glu465 (Glu366 in P-450_{cam}). Such an interaction may pull the distal helix away from the active-site region, accommodating the glutamate side chain of the mutant and generating more room in the active site for the substrate 7-ethoxycoumarin, enhancing its catalysis.

Phe313 is moderately conserved in microsomal P-450s (Gotoh & Fujii-Kuriyama, 1989). The catalytic activity (k_{cat}/K_m) of the mutant Phe313Tyr toward benzphetamine was half that of the wild type, while that toward 7-ethoxycoumarin was similar to that of the wild type. Phenylalanine is probably conserved in this location for packing reasons, as this side chain extends into a hydrophobic portion of P-450_{cam}. The replacement of Phe with Tyr may perturb protein packing interactions. Such effects could propagate into the active-site region, where they could conceivably have a greater influence on catalysis of flexible substrates (benzphetamine) than of more rigid substrates (7-ethoxycoumarin).

The mutant Ala315Ser showed an increase in catalytic activity toward benzphetamine, but a decrease toward 7-ethoxycoumarin. Residue 315 is thought to be located in the distal helix, in very close proximity to the heme plane. Again, serine is a slightly bulkier side chain than alanine and may reduce the number of conformations benzphetamine can assume, facilitating its metabolism, while interfering with the approach of the rigid 7-ethoxycoumarin to the heme iron. Of course, the oxygen atom of the serine side chain may also have electrostatic effects on the enzyme and substrate.

The mutant Thr322Ala showed enhanced catalysis against both substrates; the effect was greater with 7-ethoxycoumarin. In contrast to residue Thr319, which is near the middle (near the heme iron) of the active-site pocket in P-450_{cam}, Thr322 is located at one extreme of the active-site pocket (Figure 5) and presumably has less of an influence on substrate orientation. The effect of this mutation then appears to be a slight elongation of the active site of P-450_d, whose result seems to increase activity toward both substrates monitored. Perhaps a smaller substrate (such as acetanilide, Figure 6) would become more mobile in an enlarged active site, with a resulting decrease in catalytic activity. Low activity of the mutant toward acetanilide was in fact observed (Furuya et al., 1989).

Thus, the exact identity of conserved amino acids such as Ala315, Gly316, and Thr322 may not be extremely important for catalytic activity of eukaryotic membrane-bound P-450s. Rather, they probably together define a substantial hydrophobic pocket in which substrates of varying size, shape, and flexibility may bind. In keeping with this idea, changes in amino acid residues in the active-site area that do not preserve the overall hydrophobicity of the active site or that introduce large bulky groups into the pocket might be expected to reduce an enzymic activity of P-450_d mutants toward its entire range of substrates.

Interestingly, both k_{cat} and K_m values toward benzphetamine of all of the distal single and multiple mutants described here were lower than those of the corresponding wild-type values. Thus, it appears that all of the mutations we have made enhance the binding of benzphetamine to P-450_d, but also decrease the apparent turnover number. Unfortunately, without a reliable molecular model of P-450_d we are at a loss to explain these trends structurally.

Three of the mutations that we have made of the distal region of P-450_d (Gly316Glu, Thr319Ala, Thr322Ala) noticeably increased the catalytic activity of P-450_d toward 7-ethoxycoumarin. These results have been rationalized above by a generalized expansion of the active site, removing obstacles to the proper orientation of a large rigid substrate.

The multiple distal mutant M3, Glu318Asp, Phe325Thr, was the only multiple mutant to significantly enhance catalytic activity toward either substrate tested. In contrast to benzphetamine, which bound more tightly to most of the distal and proximal mutants than it did to wild-type P-450_d, only the K_m value of 7-ethoxycoumarin binding to the mutant M3, Glu318Asp, Phe325Thr, was much smaller than those obtained with either wild type or most of the other distal and proximal mutants. This finding is in accordance with the observation that the affinity of metyrapone to this mutant was 2 orders of magnitude higher than that to the wild type (Shimizu et al., 1989). At first glance (Figure 6), metyrapone looks more like benzphetamine than 7-ethoxycoumarin. However, the "linker" between the rings of metyrapone is half as short as that in benzphetamine; thus, metyrapone is probably intermediate between benzphetamine and 7-ethoxycoumarin in the number of its degrees of freedom. Thus, from our data presented here, the multiple distal mutant M3 seems to generaly enhance substrate binding. Phe325 appears to be too far from the active site to have much of a direct effect on substrate binding and catalysis, but the side chain of Glu318 seems to have easy access to the substrate. Perhaps the location of the carboxylate group at the end of an aspartate side chain is closer to the positions of potential hydrogen-bonding groups on various substrates. Hydrogen-bonding between enzyme and substrate may also explain the increased catalysis seen with the double mutant M3, Glu318Asp, Phe325Thr, and 7-ethoxycoumarin. On the other hand, the side chain of residue 318 could potentially interact with other charged groups away from the active site, and its effects on catalysis could be indirect.

We were not successful in simulating activities of other P-450 species solely by making mutations in the distal helix of P-450_d. Thus, the distal helix region is not the only one to contribute to the substrate specificity of P-450_{cam} and presumably of other P-450s. Consider that rat liver P-450_c

has a turnover number toward benzphetamine of 6.7 nmol min^{-1} (nmol of P-450)$^{-1}$ (Ryan et al., 1980) or 13 nmol min^{-1} (nmol of P-450)$^{-1}$ (Guengerich et al., 1982), which is higher than that (3.9 or 11 nmol min^{-1} (nmol of P-450)$^{-1}$ of wild-type rat liver P-450$_d$. If the multiple mutant designed to convert P-450$_d$ to P-450$_c$ were a good mimic of P-450$_c$, the activity of the mutant M1 (the mimic of P-450$_c$) toward benzphetamine should approach that of the wild-type P-450$_c$. However, the activity of the mutant M1 toward benzphetamine was not even half that of the wild-type P-450$_d$. Similarly, the turnover number of rabbit liver P-450$_{LM4}$ toward benzphetamine is 6.4 nmol min^{-1} (nmol of P-450)$^{-1}$ (Koop & Coon, 1979), while the multiple mutant M2 (the mimic of P-450$_{LM4}$) has a turnover number of 0.20 nmol min^{-1} (nmol of P-450)$^{-1}$.

Similar results were seen when 7-ethoxycoumarin was used as the substrate in monitoring the performances of the multiple mutants as mimics of other P-450 enzymes. Activities of the multiple mutants M1–4 toward 7-ethoxycoumarin were almost the same as or higher than that of the wild type. Turnover numbers of P-450$_c$ and P-450$_{LM4}$ toward 7-ethoxycoumarin are 27–37.3 and 3.6–0.02 nmol min^{-1} (nmol of P-450)$^{-1}$, respectively (Guengerich et al., 1982; Ryan et al., 1980; Johnson & Muller-Eberhard, 1977). Both the multiple mutants M1 (the mimic of P-450$_c$) and M2 (the mimic of P-450$_{LM4}$) have turnover numbers of 0.23 nmol min^{-1} (nmol of P-450)$^{-1}$ toward 7-ethoxycoumarin (Table II). The catalytic activity (k_{cat}/K_m) of the multiple mutant M1 toward 7-ethoxycoumarin was not even twice as high as that of the wild type. Since the apparent k_{cat} value of P-450$_c$ toward 7-ethoxycoumarin is much higher (177-fold) than that of P-450$_d$, the mutant M1 (the mimic of P-450$_c$) does rather poorly simulate the high activity of P-450$_c$ toward 7-ethoxycoumarin. Thus, substrate specificity of P-450$_d$, as mentioned above, is probably controlled by several sites in addition to the region studied here. Sligar et al. (1988) and Atkins and Sligar (1988, 1989) altered regiospecificity of P-450$_{cam}$ by changing distal amino acids such as Tyr96, Val295, and Val247 to others.

In conclusion, it seems likely that (1) the invariant Thr319 is a prerequisite for activity only toward substrates resembling benzphetamine, that is, toward flexible substrates which perhaps need conformational direction by the enzyme active site to facilitate their adoption of a catalytically correct conformation; (2) the invariant Thr319 is not a prerequisite for activity toward substrates such as 7-ethoxycoumarin which consist primarily of a rigid, fused multiple ring system and which presumably are less difficult to orient in the active site; (3) highly conserved hydrophobic residues near the P-450$_d$ active site (Ala315 and Thr322) are probably conserved not because they play a direct role in catalysis but because they are instrumental in defining the geometry and hydrophobicity of an active site capable of accommodating a number of P-450 substrates; and (4) either the distal helix is not the only effector of substrate specificity or the differences in other regions may cause a difference in conformation of the sital helix, thereby preventing it from defining the substrate specificity observed in other P-450 species. Measurements of catalytic activities of these distal mutants toward other substrates are now in progress in our laboratory.

Proximal Mutants. The mutant Lys453Glu, a mutant in which a highly conserved positive charge (Nelson & Strobel, 1988) was changed to a negative charge, was the only mutant described in this paper that was not active against either of the substrate benzphetamine or 7-ethoxycoumarin. Since this mutant also did not show activity toward other substrates such as acetanilide, zoxazolamine, and 17β-estradiol[3] (Figure 6; our unpublished results), Lys453 appears to be critical for catalytic activity of microsomal P-450s.

It has been suggested that NADPH–P-450 reductase interacts with lysine(s) that is (are) located at the enzyme surface situated on the proximal side of the heme in microsomal P-450s (Nelson & Strobel, 1988). It is conceivable that Lys453 of P-450$_d$ contacts NADPH–P-450 reductase to initiate the electron-transfer process. In comparison with the structure of P-450$_{cam}$, Lys453 is expected to be located quite close to the proximal side of the heme on the surface of the enzyme (Figure 5). It is in fact in this region that the heme approaches closest to the molecular surface, and as a result this region is considered the most attractive docking site between P-450 and its reductase. The observation that changing this residue disrupts the activity toward both substrates and electron transfer from its reductase provide a starting point for further investigations into the effects of mutations at this position as well as in its immediate environment.

It was also noted that turnover numbers and catalytic activities of the mutant Arg455Gly toward benzphetamine and 7-ethoxycoumarin were very low in comparison with those of other distal and proximal mutants and with those of the wild-type enzyme. Thus, Arg455 also appears to be important for catalytic activity. This residue is located on the surface of the molecule and very near to Lys453. However, in contrast to the positive charge conserved at Lys453 in all exogenous drug-metabolizing P-450s, a positive charge at Arg455 is only conserved in 3-methylcholanthrene-induced P-450s (Goto & Fujii-Kuriyama, 1989; Nelson & Strobel, 1988). Such charge conservation differences probably reflect evolutionary relationships and may indicate relative importance of residues. While Arg455 may aid in docking some drug-metabolizing P-450s to their corresponding reductases, Lys453 may play a similar but more universal role in all drug-metabolizing P-450s. Detailed kinetic studies on the electron-transfer process as well as on the interaction between P-450$_d$ and NADPH–P-450 reductase are in progress in our laboratory.

It has been suggested (Shimizu et al., 1988) that the invariant Arg454 in P-450$_d$ interacts with the heme propionate in much the same way as does His355 in P-450$_{cam}$ (Figure 5). It is interesting to note that the mutant Arg454His has an activity toward 7-ethoxycoumarin that is 2.5-fold higher than that of the wild-type enzyme. A difference in pK_a values between histidine and arginine may affect ionic interactions between the heme and the apoprotein and perhaps the electrostatic properties of the active site, resulting in the observed enhancement, with respect to wild type, of the activity of the mutant Arg454His toward 7-ethoxycoumarin.

The mutant Arg454Leu retained some activity toward both substrates, demonstrating that although, by analogy with the P-450$_{cam}$ structure, this arginine interacts with the heme propionate, it is clearly not essential for the activity.

The mutant Gly450Ser did not show any activity toward benzphetamine, while this mutant had activity toward 7-ethoxycoumarin as well as toward acetanilide (Furuya et al., 1989), zoxazolamine, and 17β-estradiol (Figure 6). There is no obvious structural explanation for this mutant's lack of activity toward only benzphetamine. Gly450 corresponds to Gly352 in P-450$_{cam}$, a residue that makes a close approach to the proximal side of the heme. Any mutation might be expected to push the heme toward the substrate and to reduce activity toward all substrates. Perhaps the explanation is again

[3] Activity of the mutant Lys453Glu toward 17β-estradiol described previously (Shimizu et al., 1988) is not correct.

that more flexible molecules such as benzphetamine are more sensitive to the active-site conformation than are rigid molecules like 7-ethoxycoumarin, zoxazolamine, and 17β-estradiol.

In contrast, the mutant Ile457Ser did not show any activity toward 7-ethoxycoumarin, while retaining some activity toward benzphetamine. Ile457 is also located on the proximal side of the heme plane, close to the axial cysteine ligand to the heme iron atom. Thus, a serine in this position in which hydrophobicity is conserved (Nelson & Strobel, 1988) undoubtedly has electrostatic effects on oxygen activation. We can only conclude that such effects are substrate dependent.

The turnover numbers of the proximal mutants toward benzphetamine were much lower than that of the wild type. K_m values of proximal mutants toward benzphetamine were, on the whole, also lower than that of the wild type. In contrast, no such trends were observed in the catalytic parameters of the mutants with respect to 7-ethoxycoumarin as substrate. Thus, it appears that mutations at the proximal region may cause indirect conformational and/or electrostatic changes at the distal region, which may in turn influence the activation of molecular oxygen or substrate-binding behavior.

The mutations Leu451Ser, Glu459Ala, and Ile460Ser are all located on the surface near Lys453, the region being thought to interact with NADPH–P-450 reductase. Why these mutations do not have similar effects on metabolism of different substrates is not clear. It would be expected that mutations in the reductase-interaction region would either enhance such interaction or disrupt it but that such effects would not be substrate dependent. An alternative explanation is that these surface changes perturb P-450$_d$ packing, influencing the active-site environment, which affects different substrates differently.

Aromatic Mutants.[2] All of the "aromatic region" mutations we made were near the P-450$_d$ surface, in analogy with the P-450$_{cam}$ structure. The mutant Pro427Leu had no activity toward benzphetamine. However, the activity of this mutant was enhanced toward 7-ethoxycoumarin. Similarly, k_{cat} values of the other aromatic mutants toward benzphetamine were about one-sixth that of the wild type, while those toward 7-ethoxycoumarin were similar to that of the wild type. Catalytic activities, k_{cat}/K_m, of the three aromatic mutants toward 7-ethoxycoumarin were more than twice that of the wild type. Here again we are forced to conclude that amino acids important for catalytic activity may differ depending on the substrate, even though these residues do not appear to be in the immediate vicinity of the active site.

In addition, we suggest that Phe425, Pro427, and Phe430, though they are invariant in more than 34 eukaryotic microsomal P-450 sequences (Gotoh & Fujii-Kuriyama, 1989), may not be involved in the electron-transfer process in P-450$_d$ nor may the corresponding residues be involved in catalysis in microsomal P-450s in general. It is a bit difficult to imagine that the electron-transfer path from NADPH–P-450 reductase to the enzyme active site might be substrate dependent. Residues in this aromatic region may nonetheless play a role in interactions between P-450 and its reductase. By analogy with the crystal structure of P-450$_{cam}$ and according to the membrane topology by Nelson and Strobel (1988), the aromatic region forms a loop on the cytosolic surface of the triangular P-450 molecule. The aromatic region is also just upstream, in terms of primary sequence, of the P-450 regions that may be involved in electron transfer and in contact with the proximal face of the heme. Changes in the aromatic region may thus have greater effects on the electrostatics of substrate catalysis than on substrate binding in the distal region. There is no obvious structural reason, based on our model P-450$_d$ structure, as to why the catalytic activities of aromatic region mutants are enhanced toward 7-ethoxycoumarin and not toward benzphetamine.

We also attempted to make mutants in which other amino acids of the aromatic region were changed to leucine (Trp419Leu, Pro422Leu, and Arg429Leu). However, the heme did not bind to the apoprotein of these mutants, suggesting that the aromatic region may indirectly contribute to the binding of heme in P-450$_d$. Pro422 is in a turn, and Arg429 forms hydrogen bonds across the aromatic loop to the carbonyl oxygen atoms of residues 420 and 421. Presumably all three of these residues (Trp419, Pro422, and Arg429) are important in stabilizing the tertiary structure of this region.

Conclusions. None of our mutants had significantly higher catalytic activity toward benzphetamine than did wild-type P-450$_d$; most had much lower activities. In contrast, many of the same mutants had enhanced activity toward 7-ethoxycoumarin. The most obvious structure difference between the two substrates is that benzphetamine consists of two phenyl rings joined by a linker region of four atoms, around each of which torsional rotation is possible. 7-Ethoxycoumarin, on the other hand, consists of two fused rings that are not mobile with respect to each other. Thus, we suggest that flexible substrates such as benzphetamine are more sensitive to the active-site environment of P-450s and require active-site features such as hydrophilic groups or projecting side chains to facilitate their orientation in the active site prior to catalysis. More rigid substrates have fewer possible conformations and consequently need less direction from the enzyme to assume a correct orientation for catalysis.

On the basis of our model of P-450$_d$, both the active site and substrate access channel of this enzyme appear to be lined by smaller hydrophobic amino acids than are found in corresponding positions in P-450$_{cam}$ (Nelson & Strobel, 1988; Poulos et al., 1985, 1986). Moreover, several residues in a loop including Tyr96 of P-450$_{cam}$ appear to be entirely missing in P-450$_d$. This loop closed off the substrate-binding pocket and may move to allow substrates to enter and leave the active site (Poulos et al., 1986). In addition, Tyr96 donates a hydrogen bond to the substrate camphor in P-450$_{cam}$, presumably to orient it (Poulos et al., 1985). These results lead us to suggest that the active site and substrate access channel of rat P-450$_d$ (and perhaps of all eukaryotic P-450s) are larger to accommodate the larger substrates that this enzyme metabolizes.

Added in Proof

After submitting the manuscript, we became aware of papers that reported that mutations at the distal and other sites of microsomal P-450s changed their catalytic activities and substrate specificities (Imai & Nakamura, 1988, 1989; Lindberg & Negishi, 1989).

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Quantitative Evaluation of the Contribution of Ionic Interactions to the Formation of the Thrombin–Hirudin Complex

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ABSTRACT: The effect of ionic strength on the kinetics of inhibition of human α-thrombin has been examined by using genetically engineered forms of hirudin that differed only in the number of negatively charged residues in the carboxyl-terminal region of the molecule. Analysis of the data obtained allowed the binding energy for the thrombin–hirudin complex to be divided into contributions from ionic and nonionic interactions. The contribution of nonionic interactions to the binding energy was the same for each of the forms whereas the ionic contribution varied with the charge of the molecule. Each of the negatively charged residues made an approximately equal contribution of −4 kJ mol^{-1} to the binding energy. For native hirudin, ionic interactions accounted for 32% of the binding energy at an ionic strength of zero.

Hirudin is a polypeptide inhibitor of thrombin. It has been isolated from the medicinal leech in 2 forms consisting of 65 amino acids and containing a sulfated tyrosyl residue (Bagdy et al., 1976; Dodt et al., 1984, 1986). Hirudin reacts rapidly with thrombin to form a tight complex with a dissociation constant in the femtomolar range (Stone & Hofsteenge, 1986; Braun et al., 1988a; Dodt et al., 1988). The formation of this complex involves interactions with the active site of thrombin and with additional secondary binding sites (Stone et al., 1987). In fact, the contribution to the tightness of the complex made by the binding of hirudin to the primary specificity pocket of thrombin appears to be small (Dodt et al., 1988; Braun et al., 1988a). The acidic C-terminal region of hirudin has been implicated in interactions with a secondary binding site on thrombin (Chang, 1983; Braun et al., 1988a). This negatively charged region of hirudin is thought to interact with an op-

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