Steiner, H., Jonsson, B.-H., & Lindskog, S. (1975) *Eur. J. Biochem. 59*, 253-259.
Tashian, R. E. (1989) *BioEssays 10*, 186-192.
Tu, C. K., & Silverman, D. N. (1982) *Biochemistry 21*, 6353-6360.
Tu, C. K., & Silverman, D. N. (1985) *Biochemistry 24*, 5881-5887.
Tu, C. K., Sanyal, G., Wynns, G. C., & Silverman, D. N. (1983) *J. Biol. Chem. 258*, 8867-8871.
Tu, C. K., Thomas, H. G., Wynns, G. C., & Silverman, D. N. (1986) *J. Biol. Chem. 261*, 10100-10103.
Tu, C. K., Silverman, D. N., Forsman, C., Jonsson, B. H., & Lindskog, S. (1989) *Biochemistry 28*, 7913-7918.
Tu, C. K., Paranawithana, S. R., Jewell, D. A., Tanhauser, S. M., LoGrasso, P. V., Wynns, G. C., Laipis, P. J., & Silverman, D. N. (1990) *Biochemistry 29*, 6400-6405.

Ligand Binding Studies of Engineered Cytochrome P-450$_d$ Wild Type, Proximal Mutants, and Distal Mutants†

Toru Shimizu,*,‡ Abu Jafar Md. Sadeque,‡ Gul Nahar Sadeque,‡ Masahiro Hatano,‡ and Yoshiaki Fujii-Kuriyama§
Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Katahira, Sendai 980, Japan, and Department of Chemistry, Faculty of Science, Tohoku University, Aoba, Sendai 980, Japan
Received July 31, 1990; Revised Manuscript Received November 6, 1990

ABSTRACT: Interactions of various axial ligands with cytochrome P-450$_d$ wild type, proximal mutants (Lys453Glu, Ile460Ser), and putative distal mutants (Glu318Asp, Thr319Ala, Thr322Ala) expressed in yeast were studied with optical absorption spectroscopy. P-450$_d$ wild type and all five mutants were purified essentially as the high-spin form, but the putative distal mutants contained about 5% low-spin form. Bindings of metyrapone and 4-phenylimidazole to the wild type and all mutants formed nitrogen-bound low-spin forms. In contrast, binding of 2-phenylimidazole to the wild type and most of mutants formed oxygen-bond low-spin forms except for the mutant Glu318Asp in which the nitrogen-bound low-spin form was formed. By analogy with the distal structure of P-450$_{cam}$, it was thus suggested that Glu318 of P-450$_d$, which corresponds with Asp251 of P-450$_{cam}$, somehow interacts with 2-phenylimidazole over the heme plane. Addition of 1-butanol and acetanilide, a substrate of P-450$_d$, to the wild type and mutants caused the spin change to the low-spin form. The order of dissociation constants of these oxygen ligands to P-450$_d$ was wild type > proximal mutants > putative distal mustants. Spectral analyses showed that the binding site of acetanilide is the same as that of another substrate, 7-ethoxycoumarin, in the putative distal mutants but is not the same in the wild type and proximal mutants. From these findings together with other spectral data, it was suggested that the region from Glu318 to Thr322 is located at the distal region of the heme in membrane-bound P-450$_d$ as suggested from the X-ray crystal structure of water-soluble P-450$_{cam}$ and amino acid alignments of P-450s.

Cytochrome P-450 (P-450)[1] is the heme enzyme that catalyzes monooxidation reactions of organic substrates (Sato & Omura, 1978; Ortiz de Montellano, 1986). Although the crystal structure of the water-soluble bacterial P-450$_{cam}$ is known (Poulos et al., 1985, 1986; Poulos & Howard, 1987), the tertiary structure of membrane-bound microsomal P-450s has not been understood. With appropriate alignments of amino acid sequences of membrane-bound P-450s together with that of P-450$_{cam}$, it is feasible to speculate the tertiary structure of membrane-bound P-450s to a certain extent (Nelson & Strobel, 1988; Gotoh & Fujii-Kuriyama, 1989). Furthermore, modern DNA recombinant techniques have enabled us to replace amino acids at the specific site of the huge protein molecule (Blow et al., 1986). Those techniques have become highly useful for studying the structure–function relationship of the enzyme. With the aim of understanding the structure of the heme environment of membrane-bound P-450, we first replaced amino acids of the conserved Cys in the carboxy-terminal region of the membrane-bound P-450$_d$ (Shimizu et al., 1988). It was proved that the conserved Cys in the carboxy-terminal region of the membrane-bound P-450 is the axial ligand of P-450 and that the structure of the proximal heme environment of the membrane-bound P-450 is similar to that of water-soluble P-450$_{cam}$. On the basis of the sequence alignment and the X-ray crystal structure, we further replaced amino acids at the putative distal region of P-450$_d$ (Furuya et al., 1989a,b). It was implied that the putative distal amino acids of the heme in P-450$_d$ are important for catalytic activities and/or substrate specificities. Nevertheless, the precise tertiary structure of membrane-bound P-450 is not known yet. It was thought necessary to obtain more direct structural information on this membrane-bound P-450$_d$ by conventional spectral methods.

Optical absorption spectra of P-450$_{cam}$ (Dawson et al., 1982) and microsomal P-450s (White & Coon, 1982) provided valuable information on the heme environment of this enzyme. To obtain more precise structural information on the heme

† This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (02217102) from the Ministry of Education, Science and Culture of Japan to T.S.
‡ Tohoku University, Katahira.
§ Tohoku University, Aoba.

[1] Abbreviations: P-450, cytochrome P-450; P-450$_d$, rat liver microsomal cytochrome P-450$_d$, which corresponds to P-450IA2; P-450$_{cam}$, cytochrome P-450 purified from *Pseudomonas putida* grown in the presence of camphor, which corresponds to P-450CI; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; ESR, electron spin resonance; 7-ethoxycoumarin, 7-ethoxy-2*H*-1-benzopyran-2-one; acetanilide, *N*-phenylacetamide; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; Emulgen 913, poly(oxyethylene) *p*-nonylphenyl ether containing 13.1 oxyethylene units on average; K_d, dissociation constant.

0006-2960/91/0430-1490$02.50/0

environment of the membrane-bound P-450$_d$, in the present study we purified engineered wild type, two proximal mutants, and three putative distal mutants of P-450$_d$ expressed in yeast and studied the interaction of various axial ligands with those P-450$_d$s in terms of optical absorption spectra of the Soret region. On the basis of spectral findings, we suggest here that the region Glu318–Thr322 is located in the distal site as has been implied from the sequence alignment (Nelson & Strobel, 1988; Gotoh & Fujii-Kuriyama, 1989) and the crystal structure of water-soluble P-450$_{cam}$ (Poulos et al., 1985).

Experimental Procedures

Site-directed mutageneses of P-450$_d$ and expressions of the mutant proteins in yeast were described previously (Shimizu et al., 1986, 1988, 1989; Furuya et al., 1989a,b). Yeast microsomes were prepared by crushing yeast cells with a MSK cell homogenizer (B. Braun, FRG) and by centrifugation at 77000g for 90 min. About 1.5 g of microsomal protein was suspended in 0.1 M potassium phosphate buffer (pH 7.2) containing 20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, and 0.8% (w/v) potassium cholate to make 1.5 mg of protein/mL of solution. The suspension was gently stirred for 120 min and centrifuged at 77000g for 90 min. The resultant supernatant was directly applied to a ω-amino-n-hexyl-Sepharose 4B column (1.5 g of protein/100 g wet weight of gel; column diameter, approximately 3 cm) that had been equilibrated with the same solubilizing buffer. After washing the column with solubilizing buffer, the enzyme was eluted with the same solubilizing buffer plus 0.1% (w/v) Emulgen 913. The eluted enzyme fractions had specific contents of 8–12 nmol of P-450/mg of protein. The enzyme solution was concentrated with Amicon Centriflo (Amicon) or Pellicon Lab Cassete (Millipore). Cholate was removed by changing the buffer of the enzyme solution to a 0.1 M potassium phosphate–20% glycerol–1 mM EDTA–1 mM DTT–0.1% Emulgen 913 solution (pH 7.2) with Amicon Centriflo (Sotokawa et al., 1990). For spectral experiments, Emulgen 913 was removed in the same way, making the enzyme solution 0.1 M potassium phosphate–20% glycerol–1 mM EDTA–1 mM DTT (pH 7.2).

Molar absorbance was obtained by the pyridine–hemochrome method (Theorell & Akeson, 1953). Protein concentrations were determined by the Coomassie Brilliant Blue method (Bradford, 1976). ω-Amino-n-hexyl-Sepharose 4B was prepared from Sepharose 4B (Pharmacia) according to the method of Cuatrecasas (1970). All other reagents were of the highest guanranteed grade, were purchased from Wako Pure Chemicals (Osaka), and were used without further purification. Emulgen 913 was kindly supplied by Kao-Atlas (Tokyo).

For the titration experiments, 0.2–0.8 μM P-450$_d$ solutions in 1-cm cells were used in the buffer described above. Concentrations of the free ligands were determined from the Soret absorbance of the high-spin-type oxidized or CO-reduced forms (Table I). Molar absorptivity of the nitrogen-bound and oxygen-bound forms was uniformly presumed as 1.10×10^5 M^{-1} cm^{-1}, since we often did not obtain 100% low-spin form in the present study even in the presence of excessive ligands. It should be noted that molar absorptivities depend on the coordinated atom and ligands, thus they vary in the range $(0.98–1.16) \times 10^5$ M^{-1} cm^{-1} (White & Coon, 1982; Dawson et al., 1982). This range does not essentially affect the estimation of the concentration of free ligands.

Stock solutions, 0.1–1 M, of axial ligands, such as metyrapone, 4-phenylimidazole, 2-phenylimidazole, and acetanilide, were made in methanol. We proved that methanol up to 6 M does not bind to the heme iron in the wild-type and mutant P-450$_d$s in terms of absorption spectra. Thus methanol did not perturb the present binding study at the concentration of methanol employed. For titration studies except for CO titrations, we always kept each solution at least 10 min at room temperature to reach equilibrium after the ligand was added to the solution. For CO binding studies, we kept each solution for 3 min to reach equilibrium after adding the CO stock solution. The concentration of CO in the stock solution was determined spectrophotometrically each time by titrating a solution of reduced horse heart myoglobin with the CO stock solution.

Optical absorption spectra were obtained with a Shimadzu UV spectrophotometer (UV-365) or JASCO UV spectrophotometer (Uvidec 510).

Table I: Absorption Spectra of Purified High-Spin Forms of Wild-Type and Mutant P-450$_d$s

	absorption	
	nm[a]	ϵ[b]
wild type[c]	393	1.09×10^5
	646	8.6×10^3
proximal mutants		
Lys453Glu	393	1.09×10^5
	646	8.8×10^3
Ile460Ser	393	1.21×10^5
	646	9.1×10^3
putative distal mutants		
Glu318Asp	394	1.20×10^5
	646	8.7×10^3
Thr319Ala	393	1.87×10^5
	646	9.2×10^3
Thr322Ala	393	1.27×10^5
	646	8.6×103

[a] The spectral band width was 0.5 nm under our experimental conditions, scanning rate 20 nm/min, and wavelength expansion 2 nm/cm, according to instructions of manufacturers. Experiments to determine the wavelengths for each mutant were repeated more than 10 times. [b] Molar absorptivity was expressed in M^{-1} cm^{-1}. Experiments of the pyridine–hemochrome method were repeated at least three times and their averaged values are described. Experimental errors were less than 5% or less than 10% for the Soret and visible peaks, respectively. [c] The CO-reduced form of the wild type has the Soret peak at 447 nm with a molar absorptivity of 1.20×10^5 M^{-1} cm^{-1}.

Results

Optical Absorption Spectra. Figure 1 shows optical absorption spectra of purified wild type, proximal mutants, and putative distal mutants. In Table I, absorption spectra of those P-450$_d$s are summarized. The Soret peaks of the wild-type and mutant P-450$_d$s were located at 393 nm and thus they were obtained as essentially the high-spin form in terms of the absorption spectra (Sato & Omura, 1978; Dawson et al., 1982; White & Coon, 1982). This finding is in accordance with the ESR finding at cryogenic temperature where only high-spin signals were observed for the wild type, proximal, and putative distal mutants (Sotokawa et al., 1990). However, the putative distal mutants had a small shoulder around 416 nm, suggesting that about 5% low-spin form was present in their preparation (Figure 1C). The shoulder around 416 nm was decreased by adding 1 mM 7-ethoxycoumarin, one of substrates of P-450$_d$.

Interactions with Nitrogenous Ligands. It is known (White & Coon, 1982; Dawson et al., 1982) that oxygen-bound low-spin P-450 complexes have the Soret peak at relatively lower wavelengths down to 416 nm, while nitrogen-bound low-spin complexes have the peak at higher wavelengths of 419–423 nm. Addition of nitrogenous ligands such as metyrapone, 4-phenylimidazole, and 2-phenylimidazole to the P-450$_d$ wild type and mutants caused the spin change from the high-spin state to the low-spin state (Figure 2, Table II). Low-spin forms of the wild type and most of the mutants in the presence

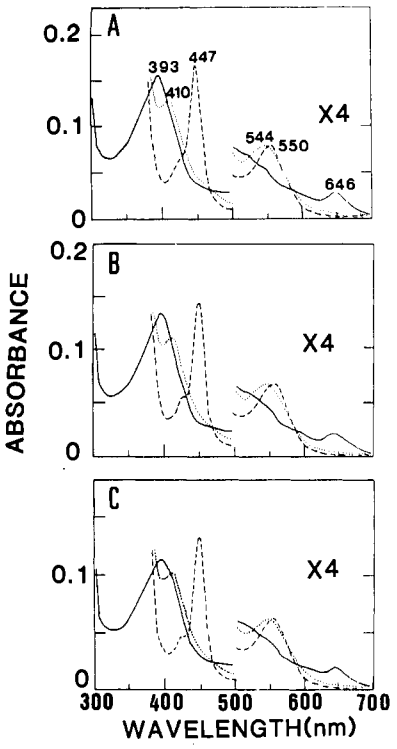

FIGURE 1: Absorption spectra of oxidized (—), reduced (···), and CO-reduced (---) forms of purified wild type (A), mutant Lys453Glu (B), and mutant Glu318Asp (C). Peak positions were essentially the same for the wild type and mutants. Putative distal mutants such as Glu318Asp have a shoulder around 416 nm.

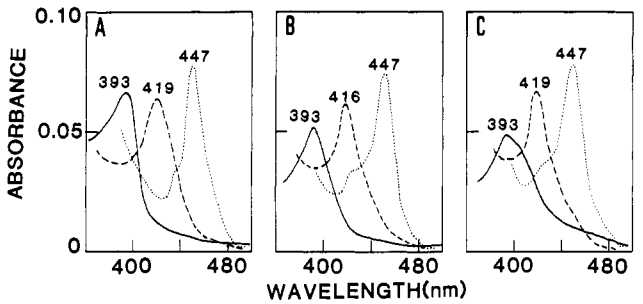

FIGURE 2: Soret absorption spectra of low-spin forms (---) of the wild type (0.60 μM) (A) in the presence of 0.3 mM metyrapone, mutant Ile460Ser (0.43 μM) (B) in the presence of 4 mM 2-phenylimidazole, and mutant Glu318Asp (0.43 μM) (C) in the presence of 0.3 mM 2-phenylimidazole. Oxidized forms (—) and CO-reduced forms (···) are shown for reference, showing that ligand-bound low-spin forms are not denatured from CO-reduced forms.

of metyrapone had the Soret peak at 419 nm, indicating that the nitrogen-bound low-spin complex is formed. However, the mutant Thr319Ala had the Soret peak at 417 nm, which is a little lower than those of other complexes. Similarly low-spin forms of the wild type and all mutants in the presence of 4-phenylimidazole had the Soret peaks at 420–423 nm, suggesting that the nitrogen atom of the ligand directly binds to the heme iron in those low-spin complexes. In contrast, the low-spin complexes of the wild type and most of mutants in the presence of 2-phenylimidazole had the Soret peak at 416 nm except for the mutant Glu318Asp in which the Soret peak is located at 419 nm. Thus, it appears that the nitrogen atom of 2-phenylimidazole does not directly coordinate to the heme iron for the wild type, two proximal mutants, and putative distal mutants, Thr319Ala and Thr322Ala as for P-450$_{cam}$ (Poulos & Howard, 1987), while the nitrogen atom of 2-phenylimidazole binds directly to the heme iron in the mutant Glu318Asp.

Table II: Soret Absorption Spectra of Low-Spin Forms of the Wild-Type and Mutant P-450$_d$s[a]

	axial ligands				
	Metyr[b]	4-PheIm[c]	2-PheIm[d]	BuOH[e]	Acet[f]
wild type	419	423	416	416	416
proximal mutants					
Lys453Glu	419	422	416	416	416
Ile460Ser	419	423	416	416	416
putative distal mutants					
Glu318Asp	419	422	419	416	416
Thr319Ala	417	420	416	416	416
Thr322Ala	419	422	416	416	416

[a] Wavelengths of the Soret peaks are expressed in nanometers. The spectral band width was 0.5 nm. Other spectral conditions were the same as in Table I. Experiments to determine the wavelength for each mutant were repeated more than six times. [b] Metyrapone. [c] 4-Phenylimidazole [d] 2-Phenylimidazole. [e] 1-Butanol. [f] Acetanilide.

Table III: Dissociation Constants (K_d) of Nitrogenous Axial Ligands for the Wild-Type and Mutant P-450$_d$s[a]

	axial ligands		
	Mety[b]	4-PheIm[c]	2-PheIm[d]
wild type	20.3	10.3	500
proximal mutants			
Lys453Glu	14.6	10.2	12700
Ile460Ser	9.5	9.3	860
putative distal mutants			
Glu318Asp	11.3	6.6	68.9
Thr319Ala	11.9	20.7	1040
Thr322Ala	18.5	24.4	1460

[a] Dissociation constants (K_d) are expressed in micromolar units. Experiments were repeated at least three times and their averaged values are described. Experimental errors were less than 20%. [b] Metyrapone. [c] 4-Phenylimidazole. [d] 2-Phenylimidazole.

The double-reciprocal plot of the Soret spectral change around 416–423 nm caused by adding the axial ligand versus the concentration of the free ligand formed a straight line (data not shown). From the Soret spectral change, it is clear that the 1:1 ligand–heme complex is formed by adding the axial ligand for the wild type and all mutants studied here. The calculated dissociation constants (K_d) of the axial ligands to the P-450$_d$ wild type and mutants are summarized in Table III. No remarkable difference in the K_d values among the wild type and mutants was observed for the metyrapone and 4-phenylimidazole bindings. For the 2-phenylimidazole binding, in contrast, the binding ability to the mutant Glu318Asp was much higher than that to the wild type, while that to the mutant Lys453Glu was much lower than that of the wild type.

The low-spin complex of the 2-phenylimidazole–mutant Glu318Asp solution seems likely to form the nitrogen–heme complex. This is in contrast with those of 2-phenylimidazole–wild type solution in which the oxygen–heme complex must be formed. To test the binding site of 2-phenylimidazole in the wild-type and mutant Glu318Asp, bindings of 2-phenylimidazole in the absence and presence of 4-phenylimidazole were studied. From spectral findings (data not shown), it was suggested that the binding site of 2-phenylimidazole is not competitive with that of 4-phenylimidazole in the wild type, while that of 2-phenylimidazole is competitive with that of 4-phenylimidazole in the mutant Glu318Asp.

Interactions with Oxygenous Ligands. Binding of 1-butanol to the wild-type and mutant P-450$_d$s caused small Soret spectral change from the high-spin state to the low-spin state (Figure 3A–C) (Table II). The Soret peaks at 416 nm of the

Table IV: Dissociation Constants (K_d) of 1-Butanol and Acetanilide for the Wild-Type and Mutant P-450$_d$s[a]

	1-butanol[b] (M)	+7-Eth[c] (M)	Acet[d] (mM)	+7-Eth[e]	K_i[f] (mM)
wild type	0.83	1.00	0.111	noncomp[g]	0.71
proximal mutants					
Lys453Glu	0.25	0.25	0.043	uncomp[h]	
Ile460Ser	0.27	0.50	0.048	uncomp[h]	
putative distal mutants					
Glu318Asp	0.05	0.06	0.007	comp[i]	0.07
Thr319Ala	0.05	0.02	0.014	comp[i]	0.05
Thr322Ala	0.09	0.07	0.005	comp[i]	0.04

[a] Experiments for dissociation constants (K_d) were repeated at least three times and their averaged values are described. Experimental errors were less than 20%. [b] Dissociation constants (K_d) for 1-butanol. [c] K_d values of 1-butanol in the presence of 2 mM 7-ethoxycoumarin. [d] K_d values of acetanilide. [e] Competitive manner of acetanilide binding against 7-ethoxycoumarin. [f] Inhibition constants against 7-ethoxycoumarin. [g] Noncompetitive binding of acetanilide with 7-ethoxycoumarin. [h] Uncompetitive binding of acetanilide with 7-ethoxycoumarin. [i] Competitive binding of acetanilide with 7-ethoxycoumarin.

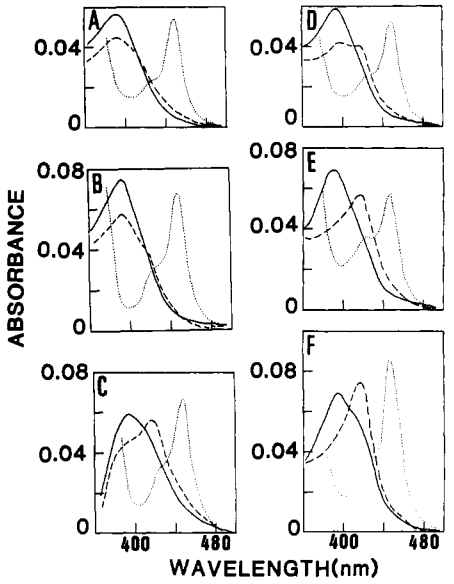

FIGURE 3: Soret absorption spectra (---) of the oxidized wild type (0.51 μM) (A) in the presence of 107 mM 1-butanol, mutant Ile460Ser (0.68 μM) (B) in the presence of 25 mM 1-butanol, mutant Glu318Asp (0.52 μM) (C) in the presence of 34 mM 1-butanol, wild type (0.54 μM) (D) in the presence of 45 mM acetanilide, mutant Lys453Glu (0.58 μM) (E) in the presence of 50 mM acetanilide, and mutant Glu318Asp (0.58 μM) (F) in the presence of 18 mM acetanilide. Oxidized high-spin forms in the absence of the ligand (—) and CO-reduced forms in the presence of the ligand (···) are also shown for reference, indicating that the wild type and mutants were not denatured by adding excess ligands. Adding more ligands to each P-450$_d$ solution remarkably increased the peak at 420 nm of the CO-reduced form, which is indicative of a denatured form.

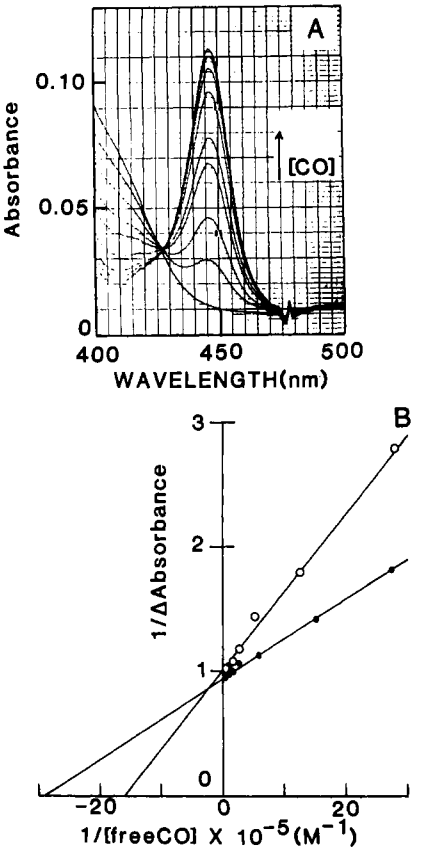

FIGURE 4: Soret absorption spectral change of reduced mutant Lys453Glu by adding CO (A). Double-reciprocal plots of the Soret spectral change at 447 nm versus the concentration of free CO in the absence (○) and presence (●) of 2 mM 7-ethoxycoumarin.

1-butanol–P-450$_d$ solutions indicate that the oxygen–heme bond was formed for the low-spin complexes. K_d values of 1-butanol for the wild type and mutants are summarized in Table IV. It was shown that 1-butanol is more accessible to distal mutants than to the wild type and proximal mutants.

Acetanilide is a substrate of P-450$_d$. Addition of acetanilide to the wild type caused the Soret spectral change from the high-spin state to the low-spin state to a small extent (Figure 3D). Adding acetanilide to the proximal and distal mutants caused a more remarkable spin change from the high-spin state to the low-spin state (Figure 3E,F). From the position of the Soret spectral peak at 416 nm for the wild type and mutants, it appears that the oxygen atom coordinates to the heme iron in those P-450$_d$ (Dawson et al., 1982; White & Coon, 1982). Double-reciprocal plots of the Soret spectral change at 416 nm by adding acetanilide versus the concentration of free acetanilide formed a straight line for the wild type and all mutants (data not shown). Thus it appeared that acetanilide forms the 1:1 ligand–heme complex in the wild type and mutant P-450$_d$s. Table IV summarizes the K_d values of acetanilide for the wild type and mutants. As observed for the binding of 1-butanol, acetanilide is more accessible to the distal mutants than to the wild type and proximal mutants. Since this is the first evidence that the substrate, acetanilide, binds directly the heme of P-450, we tested whether acetanilide binds to the same site as another substrate, 7-ethoxycoumarin. From competitive binding studies (data not shown) in the presence of 7-ethoxycoumarin, it was suggested that the binding site of acetanilide is different from that of 7-ethoxycoumarin for the wild type, while that of acetanilide is the same as that of 7-ethoxycoumarin for the putative distal mutants. For proximal mutants, however, the binding fashion of both substrates could not be clearly explained.

Binding of CO to the Reduced Form. Binding of CO to the reduced wild type and mutants was studied (Figure 4, Table V). It was found that CO is more accessible to the putative distal and proximal muants than to the wild type and is more accessible to the distal mutants than to the proximal

Table V: Dissociation Constants (K_d) of CO for the Reduced Wild-Type and Mutant P-450$_d$s[a]

	no substrates	+7-Ethoxy[b]	+Acet[c]
wild type	1.81	1.53	2.75
proximal mutants			
Lys453Glu	0.63	0.34	0.55
Ile460Ser	0.48	0.26	0.38
putative distal mutants			
Glu318Asp	0.30	0.20	0.19
Thr319Ala	0.21	0.11	0.18
Thr322Ala	0.20	0.12	0.19

[a] Dissociation constants (K_d) are expressed in micromolar units. Experiments were repeated at least three times and their averaged values are described. Experiments were repeated at least three times and their averaged values are described. Experimental errors were less than 20%. [b] K_d values in the presence of 2 mM 7-ethoxycoumarin. [c] K_d values in the presence of 20 mM acetanilide.

mutants. However, no remarkable effect of substrates, acetanilide and 7-ethoxycoumarin, on the CO affinity to P-450$_d$ was observed.

Discussion

In our previous paper, we reported that the Lys453Glu mutant is universally inactive toward many substrates (Furuya et al., 1989a,b). We suggested that Lys453, which is well conserved for microsomal P-450s and is located at the proximal site of the heme, perhaps participates in forming an intermolecular electron-transfer complex with cytochrome P-450 reductase (Furuya et al., 1989b). Thus we selected the Lys453Glu mutant for studying the heme environment of P-450 in this study. The Ile460Ser mutant was selected as another proximal mutant because this mutant efficiently expressed in yeast and was one of the most stable mutants against solubilization of yeast microsomes by the detergent.

Glu318 of P-450$_d$ of the putative distal region is highly conserved for all P-450s as either Glu or Asp (Nelson & Strobel, 1988). Glu318 of P-450$_d$ corresponds to Asp251 in P-450$_{cam}$ (Poulos et al., 1985; Nelson & Strobel, 1988). The X-ray crystal structure of P-450$_{cam}$ indicates that Asp251 interacts with imidazole of 2-phenylimidazole through a water molecule, preventing its direct coordination to the heme in P-450$_{cam}$ (Poulos & Howard, 1987). We thus thought that the Glu318Asp mutant of P-450$_d$ is worth studying spectrometrically to understand the heme environment of P-450$_d$. Thr319 is highly conserved for P-450s and is suggested to be important for the catalytic activity and/or the activation of molecular oxygen (Poulos et al., 1985; Furuya et al., 1989a,b; Imai & Nakamura, 1989; Imai et al., 1989; Martinis et al., 1989). Thr322 is also conserved in the "threonine cluster" of eukaryotic P-450s (Nelson & Strobel, 1988; Gotoh & Fujii-Kuriyama, 1989). Thus we selected the two mutants Thr319Ala and Thr322Ala for studying the putative distal environment of P-450$_d$. Those putative mutant proteins were also fairly stable against the solubilization of the microsomes.

Catalytic activities toward benzphetamine and acetanilide of these P-450$_d$ mutants were much lower than those of the wild type (Furuya et al., 1989a,b). However, no clear difference in activity between the proximal mutants and the putative distal mutants was observed. In addition, catalytic activities toward 7-ethoxycoumarin of the present mutants were comparable to that of the wild type except for the Lys453Glu mutant, which had no activities toward this and other substrates (Furuya et al., 1989b). Thus, again we have not obtained clear evidence that the region Glu318–Thr322 is located at the distal site of P-450$_d$ from catalytic activities. Therefore, it would be worthwhile to establish the identity of the distal and proximal sites of this membrane-bound protein.

Optical Absorption Spectra. The wild type and all mutants described here were purified as the high-spin form as observed for native rat liver P-450$_d$ (Ryan et al., 1980). ESR spectra at liquid helium temperature for those purified mutants were also of the high-spin form (Sotokawa et al., 1990). Yeast harboring the mutant DNA had high-spin signals as well (Shimizu et al., 1988; our unpublished observations). Thus we conclude that the mutant proteins studied here were expressed in yeast as the high-spin form and that the mutations did not largely change the heme environment of P-450$_d$.

It is noticeable that only putative distal mutants contained small quantity of the low-spin form. Most of other putative distal mutants contained 5–10% low-spin form as well (our unpublished observations). Thus it is suggested that mutations at the putative distal site subtly change the heme environment and/or destroy partially the important ternary structure for keeping the heme iron in the high-spin state. It is conceivable that a tight space over the heme plane, into which the water molecule cannot be squeezed (Sotokawa et al., 1990), may be released by the mutations at the putative distal site in P-450$_d$. Binding of 7-ethoxycoumarin decreased the low-spin content of the putative distal mutants. Substrate binding to the low-spin P-450 will result in displacement of bound water from the heme iron (Sato & Omura, 1978; Ortiz de Montellano, 1986; Poulos et al., 1985).

Interactions with Nitrogenous Ligands. Bindings of metyrapone to the wild-type and mutant P-450$_d$s formed mostly nitrogen-bound low-spin complexes as expected from previous studies of other P-450s (Peterson et al., 1971; White & Coon, 1982; Dawson et al., 1982). However, it appears that the low-spin-type Thr319Ala mutant in the presence of metyrapone is not a nitrogen-bound form but perhaps an oxygen-bound form from the Soret absorption peak (Table II). Unfortunately, at present we cannot conclude the coordination of the oxygen atom to the heme iron of this mutant, since spectral differences of this solution from others are rather small. Similar unusual coordination of the axial ligand to the putative distal mutant was observed. Namely, low-spin forms of 2-phenylimidazole-bound wild type and proximal mutants are the oxygen-bound form, but the putative distal mutant Glu318Asp in the presence of 2-phenylimidazole is a nitrogen-bound low-spin form (Table II). Thus, mutations only at the putative distal site of P-450$_d$ changed the binding fashion of the axial ligands coordinated to the heme of P-450$_d$. Glu318 corresponds to Asp251 in P-450$_{cam}$ (Nelson & Strobel, 1988; Poulos et al., 1985), which interacts with the nitrogen atom of 2-phenylimidazole through a water molecule. As a result, the 2-phenylimidazole–P-450$_{cam}$ complex in the low-spin state has another water molecule as an axial ligand (Poulos & Howard, 1987). The present spectral findings indicate that Glu318 of P-450$_d$ somehow interacts with 2-phenylimidazole and interferes the direct coordination of the imidazole nitrogen of this ligand to the heme iron as observed for P-450$_{cam}$ (Poulos & Howard, 1987; Dawson et al., 1982). In the 2-phenylimidazole–mutant Glu318Asp complex, the imidazole nitrogen will directly coordinate to the heme iron. Subtle structural difference in the heme environment, perhaps in the distal region, between the wild-type P-450$_d$ and the Glu318Asp mutant will alter the binding fashion of 2-phenylimidazole. This structural change of the distal site is also suggested by the competitive titration experiments of 2-phenylimidazole with 4-phenylimidazole for the wild type and the Glu318Asp mutant in that the structure of the binding site of 2-phenylimidazole is changed. However, we cannot totally rule out

the possibility that the mutation at Glu318 allowed a coordination of an amino acid residue in the nearest neighbor without knowing the crystal structure of this complex.

For P-450$_d$ mutants in microsomes, metyrapone and 4-phenylimidazole are more accessible to putative distal mutants than to the wild type and the proximal mutants (Shimizu et al., 1989; our unpublished results). However, K_d values of metyrapone and 4-phenylimidazole to the purified wild type and mutants were not largely different from each other (Table III). In contrast, K_d values of 2-phenylimidazole were rather diversed. Especially the K_d value of the Glu318Asp mutant was much lower than that of the wild type. Thus, it is suggested that a subtle structural change of the distal site of P-450$_d$, which was perhaps caused by widening of the heme crevice, enhanced the direct binding of 2-phenylimidazole to the heme in the Glu318Asp mutant. However, the K_d value of 2-phenylimidazole for the Lys453Glu mutant was much larger than that of the wild type. We cannot explain at present this indirect effect of the proximal mutation Lys453Glu on the K_d value of 2-phenylimidazole. Examination of a molecular model of P-450$_d$ generated by replacing amino acid side chains in the crystal structure of P-450$_{cam}$ (Furuya et al., 1989b) suggests that the carboxylate group of Glu, newly produced by the mutation, may stick out of the protein surface. It might hinder the binding of 2-phenylimidazole to the Lys453Glu mutant.

Therefore, a concluding remark in this section is that the putative distal site is located at the distal site of P-450$_d$ on the basis of the spectral findings on the coordination of the nitrogen ligands to the P-450$_d$ mutants. Similarity of the distal heme environment for all P-450s will be emphasized here (Poulos et al., 1985; Nelson & Strobel, 1988; Gotoh & Fujii-Kuriyama, 1989).

Interactions with Oxygenous Ligands. Alcohol or carboxylate bindings to heme proteins (Muhoberac & Brill, 1980) and P-450 (White & Coon, 1982; Dawson et al., 1982; Sono & Dawson, 1982) give rise to characteristic optical absorption spectra. The present spectral study (Figure 3, Table IV) certainly shows that 1-butanol and acetanilide are more accessible to the putative distal mutants than to the proximal mutants and the wild type. The competitive binding experiments (data not shown) indicate that a subtle structural change caused by the putative distal mutations produced a new binding site common for both 7-ethoxycoumarin and acetanilide. Thus, it is again suggested here that the putative distal site of P-450$_d$ is located at the distal site of the heme of this enzyme. The binding site of 1-butanol will not be the binding site of the substrate, 7-ethoxycoumarin. Mutations at the proximal site indirectly change the protein structure of the distant distal site and/or ligand binding path to a certain extent, which may change the K_d values of oxygenous ligands. It is also possible that the oxygen atom of water is the distal ligand in the 1-butanol- and acetanilide-bound P-450$_d$.

Binding of CO to the Reduced Form. It is reasonable that a subtle structural change or destruction of the distal site enhanced the CO binding more remarkably than that of the proximal site (Table V). Nevertheless, the K_d values of CO to the mutants were not largely influenced by substrates (Table V). The CO molecule is a linear ligand. It is reported that bindings of linear ligands to the heme in the heme protein are dominated by electrostatic effects not by steric effects (Smith & McLendon, 1980). Thus a structural change caused by substrates may not be electrostatically so significant as to affect the K_d values of CO for the P-450$_d$ wild type and mutants, in contrast with P-450$_{cam}$ (Peterson & Griffin, 1972). Ligation kinetics may be more influenced by the substrate binding.

Alternatively it is conceivable that the space of the distal site of native P-450$_d$ is so narrow and/or so tight as to prevent binding of the water molecule or substrates above the heme plane (Sotokawa et al., 1990). Thus the binding of the substrate will not largely influence the distal environment. This conjecture was partially verified by the binding study of rather simple or linear axial ligands such as 1-butanol (Table IV) and CO (Table V) in that the bindings of these ligands were not affected by the substrate. When the axial ligand is larger or more complicated, for example, metyrapone, 4-phenylimidazole, 2-phenylimidazole, and acetanilide, the K_d values were influenced by the substrate (our unpublished results). Perhaps these ligands will bind simultaneously to both the heme and a hydrophobic substrate binding site, which may be the reason why the K_d values of these ligands are scattered (Table III).

Mutations at the remote proximal site indirectly change the protein structure of the CO binding path and/or site, which may lead to the certain changes of the K_d values of CO.

Conclusions. On the basis of spectral findings of the wild type and several mutants of the proximal and putative distal sites, we suggest that the region at least from Glu318 to Thr322 is located at the distal region of the heme in P-450$_d$. Mutations at the remote proximal site influenced the ligand binding less markedly than those at the distal site. These findings provide a strong contrast in the effect of changes in the protein structure on the distal and proximal sides on ligand binding behavior. Recent studies on the structure of membrane-bound P-450s all suggested that the whole ternary structure of membrane-bound P-450s is almost the same as that of water-soluble P-450$_{cam}$ (Nelson & Strobel, 1988, 1989; Edwards et al., 1989; Vergeres et al., 1989; Brown & Black, 1989; Tretiakov et al., 1989; Goto & Fujii-Kuriyama, 1989). The present optical absorption study of the P-450$_d$ mutants is the first paper that experimentally identified the location of the distal site of the membrane-bound P-450. Especially the direct participation of Glu318 in ligand binding should be emphasized here. It should be noted here that site-directed mutageneses of a substrate-interacting site of P-450$_{cam}$ remarkably influenced the regiospecificity and the spin state (Atkins & Sligar, 1989; Di Primo et al., 1990).

It is to be reminded that in the absence of a determined three-dimensional structure, one cannot reach a more concrete conclusion of the protein structure. For example, Lindberg and Negishi (1989) changed the substrate specificity of a microsomal P-450 by site-directed mutagenesis at a site that is presumed to be at a distance from the active site of the P-450 on the basis of structural predictions. The folding of proteins into a tightly packed three-dimensional structure is complicated and thus small changes in the packing amino acid residues may result in substantial changes at a distance in the protein structure. Certain changes in the ligand binding parameters caused by the proximal mutations in the present study may reflect these factors. This may explain partially why catalytic activities of the distal mutants toward several substrates are not much different from those of the proximal mutants (Furuya et al., 1989a,b).

Registry No. P-450, 9035-51-2; L-Lys, 56-87-1; L-Ile, 73-32-5; L-Glu, 56-86-0; L-Thr, 72-19-5; Metyr, 54-36-4; 4-PheIm, 670-95-1; 2-PheIm, 670-96-2; BuOH, 71-36-3; Acet, 103-84-4; Fe, 7439-89-6; carbon monoxide, 630-08-0.

References

Atkins, W. M., & Sligar, S. G. (1989) *J. Am. Chem. Soc. 111*, 2715–2717.

Blow, D. M., Fersht, A. R., & Winter, G., Eds. (1986) *Design, Construction and Properties of Novel Protein Molecules*, The Royal Society, London.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Brown, C. A., & Black, S. D. (1989) *J. Biol. Chem. 264*, 4442–4449.
Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059–3065.
Dawson, J. H., Anderson, L. A., & Sono, M. (1982) *J. Biol. Chem. 257*, 3606–3617.
Di Primo, C., Hui Bon Hoa, G., Douzou, P., & Sligar, S. (1990) *J. Biol. Chem. 265*, 5361–5363.
Edwards, R. J., Murray, B. P., Boobis, A. R., & Davies, D. S. (1989) *Biochemistry 28*, 3762–3770.
Furuya, H., Shimizu, T., Hatano, M., & Fujii-Kuriyama, Y. (1989a) *Biochem. Biophys. Res. Commun. 160*, 669–676.
Furuya, H., Shimizu, T., Hirano, K., Hatano, M., Fujii-Kuriyama, Y., Raag, R., & Poulos, T. L. (1989b) *Biochemistry 28*, 6848–6857.
Gotoh, O., & Fujii-Kuriyama, Y. (1989) in *Frontiers in Biotransformation* (Ruckpaul, K., & Rein, H., Eds.) Vol. 1, pp 195–234, Akademie-Verlag, Berlin, and Taylor & Francis, London.
Imai, Y., & Nakamura, M. (1989) *Biochem. Biophys. Res. Commun. 158*, 717–722.
Imai, M., Shimada, H., Watanabe, Y., Matsushima-Hibiya, Y., Makino, R., Koga, H., Horiuchi, T., & Ishimura, Y. (1989) *Proc. Nat. Acad. Sci. U.S.A. 86*, 7823–7827.
Lindberg, R. L., & Negishi, M. (1989) *Nature 339*, 632–634.
Martinis, S. A., Atkins, W. M., Stayton, P. S., & Sligar, S. G. (1989) *J. Am. Chem. Soc. 111*, 9252–9253.
Muhoberac, B. B., & Brill, A. S. (1980) *Biochemistry 19*, 5157–5167.
Nelson, D. R., & Strobel, H. W. (1988) *J. Biol. Chem. 263*, 6038–6050.
Nelson, D. R., & Strobel, H. W. (1989) *Biochemistry 28*, 656–660.
Ortiz de Montellano, P. R., Ed. (1986) *Cytochrome P-450, Structure, Mechanism and Biochemistry*, Plenum, New York.
Peterson, J. A., & Griffin, B. W. (1972) *Arch. Biochem. Biophys. 151*, 427–433.
Peterson, J. A., Ullrich, V., & Hildebrandt, A. G. (1971) *Arch. Biochem. Biophys. 145*, 531–542.
Poulos, T. L., & Howard, A. J. (1987) *Biochemistry 26*, 8165–8174.
Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., & Kraut, J. (1985) *J. Biol. Chem. 260*, 16122–16130.
Poulos, T. L., Finzel, B. C., & Howard, A. J. (1986) *Biochemistry 25*, 5314–5322.
Ryan, D. E., Thomas, P. E., & Levin, W. (1980) *J. Biol. Chem. 255*, 7941–7955.
Sato, R., & Omura, T., Eds. (1978) *Cytochrome P-450*, Kodansha, Tokyo, and Academic Press, New York.
Shimizu, T., Sogawa, K., Fujii-Kuriyama, Y., Takahashi, M., Ogoma, Y., & Hatano, M. (1986) *FEBS Lett. 207*, 217–221.
Shimizu, T., Hirano, K., Takahashi, M., Hatano, M., & Fujii-Kuriyama, Y. (1988) *Biochemistry 27*, 4138–4141.
Shimizu, T., Sadeque, A. J. M., Hatano, M., & Fujii-Kuriyama, Y. (1989) *Biochim. Biophys. Acta 995*, 116–121.
Smith, M. C., & McLendon, G. (1980) *J. Am. Chem. Soc. 102*, 5666–5670.
Sono, M., & Dawson, J. H. (1982) *J. Biol. Chem. 257*, 5496–5502.
Sotokawa, H., Shimizu, T., Furuya, H., Sadeque, A. J. M., Hatano, M., Ohba, Y., Iwaizumi, M., & Fujii-Kuriyama, Y. (1990) *Biochim. Biophys. Acta 995*, 116–121.
Theorell, K. G., & Akeson, A. (1953) *Acta Chem. Scand. 7*, 1284–1287.
Tretiakov, V. E., Degtyarenko, K. N., Uvarov, V. Y., & Archakov, A. I. (1989) *Arch. Biochem. Biophys. 275*, 429–439.
Vergeres, G., Winterhalter, K. H., & Richter, C. (1989) *Biochemistry 28*, 3650–3655.
White, R. E., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 3073–3083.