Absorption Spectral Study of Cytochrome $P450_d$–Phenyl Isocyanide Complexes: Effects of Mutations at the Putative Distal Site on the Conformational Stability†

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Received June 11, 1991; Revised Manuscript Received September 5, 1991

ABSTRACT: Interactions of phenyl isocyanide (PheNC) with purified engineered cytochrome $P450_d$ wild type and putative distal mutants, Glu318Asp and Glu318Ala, were studied with optical absorption spectra. The wild type and the mutant Glu318Asp were purified as the high-spin state, while the mutant Glu318Ala was purified as the oxygen-bound low-spin form. Thus, it is suggested that Glu318 is important to make the appropriate heme environment of $P450_d$. Spectral dissociation constants (0.19–0.39 mM) of the ligand for the ferric mutants were lower than that (0.74 mM) of the wild type. These dissociation constants were changed by adding a substrate, 7-ethoxycoumarin. The reduced wild type–PheNC complex showed a Soret peak at 451 nm, while the reduced mutant–PheNC complexes showed two peaks at 451 and 423 nm. The 451-nm peak of the complexes decreased with the concomitant increase of a new peak at 433 nm at room temperature. Thus, it was suggested that $P450_d$ can take two conformationally different forms from the characteristic spectral features. The Soret spectral conversions which followed the first-order kinetics were analyzed by changing the temperature. The activation energy (69 kcal/mol) for the conversion for the wild type was higher than those (37–50 kcal/mol) for the mutants. The activation energy for the wild type further increased (by 55%) by adding the substrate, while those for the mutants were essentially unchanged by adding the substrate. We discuss the important role of Glu318 at the putative distal site of $P450_d$ in the packing or the conformational stability of the putative distal site of the $P450_d$ molecule.

The cytochrome P450 (P450)[1] is one of the superfamily of heme-containing monooxygenases which catalyze many types of biotransformation reactions of organic substrates in living organisms (Sato & Omura, 1978; Nebert & Gonzalez, 1987). Significant progress in understanding the P450 structure–function relationship has been made on the basis of the X-ray crystal structure of water-soluble $P450_{cam}$ (Poulos et al., 1985, 1987) and alignments of amino acids of P450s (Nelson & Strobel, 1988, 1989; Gotoh & Fujii-Kuriyama, 1988). By combining this knowledge and site-directed mutagenesis techniques, the structure of membrane-bound $P450_d$ was partially elucidated with respect to the heme incorporation (Shimizu et al., 1988) and the structure of putative distal site (Furuya et al., 1989; Shimizu et al., 1991a,b). It was found that the putative distal amino acids of the heme in $P450_d$ are important for the catalytic activities and ligand interactions. Especially, the role of Glu318 of $P450_d$ in the binding of external axial ligands was noted (Shimizu et al., 1991a,b). This position, Glu318 for $P450_d$, is highly conserved for all P450s as Glu or Asp (Nelson & Strobel, 1988, 1989; Gotoh & Fujii-Kuriyama, 1989) and corresponds to Asp251 in $P450_{cam}$. The crystal structure of $P450_{cam}$ indicates that Asp251 forms ionic bridges with Arg186 or Lys178 to make a heme cavity appropriate or necessary for the catalytic function of $P450_{cam}$ (Poulos et al., 1985, 1987). Thus, it seems that Glu318 of $P450_d$ also may form a similar ionic bridge(s) with Arg or Lys to make the distal pocket that is necessary for the catalytic function of $P450_d$. Unfortunately, the structure of mammalian P450s has not been known. To get more information on the role of Glu318 in the structure–function relationships of mammalian P450s, it was thought necessary to study the heme environment of $P450_d$ further by using another ligand for Glu318 mutants of $P450_d$.

Isocyanides are known as ligands of P450 (Imai & Sato, 1966; Ichikawa & Yamano, 1968) and serve as powerful tools for elucidating the structure of the heme surroundings of P450 and other heme proteins (Mims et al., 1983). For example, the absorption spectra of the ferrous P450–phenyl isocyanide complexes exhibit two Soret peaks, and their relative intensities are sensitive to conformational changes around the heme moiety (Imai & Sato, 1968; Tsubaki et al., 1989).

We report here how point mutations at Glu318 in the putative distal region of $P450_d$ influence the interaction of phenyl isocyanide with $P450_d$ and the heme environment of $P450_d$. Analyses of Soret bands for the complexes allowed us to evaluate the energy of structural rearrangements of $P450_d$. We discuss the important role of Glu318 in the packing of the putative distal site of the $P450_d$ molecule by referring to the X-ray crystal structure of $P450_{cam}$ (Poulos et al., 1985, 1987)

† This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

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[1] Abbreviations: P450, cytochrome P450; $P450_d$, rat liver cytochrome $P450_d$, which corresponds to P450IA2; $P450_{scc}$, adrenal mitochondrial cytochrome P450 participating in cholesterol side-chain cleavage reaction, which corresponds to P450XIIA1; $P450_{cam}$, cytochrome P450 purified from *Pseudomonas putida* grown in the presence of camphor, which correspond to P450CI; $P450_{lin}$, cytochrome P450 purified from *Pseudomonas incognita* grown in the presence of linalool, which corresponds to P450CI; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; 7-ethoxycoumarin, 7-ethoxy-2*H*-1-benzopyran-2-one; Emulgen 913, poly(oxyethylene) *p*-nonylphenyl ether containing 13.1 oxyethylene units on average; K_s, spectral dissociation constant; ΔA_{max}, maximal amplitude of spectral changes.

0006-2960/91/0430-11206$02.50/0

and the alignment of amino acid sequences of P450s (Nelson & Strobel, 1988, 1989).

Experimental Procedures

Site-directed mutagenesis of $P450_d$ and expressions of the mutant proteins in yeast were carried out as described previously (Shimizu et al., 1988, 1991a,b; Furuya et al., 1989). Preparation of yeast microsomes and purification of $P450_d$s were performed as described previously (Shimizu et al., 1991a).

The enzyme solutions were concentrated with Amicon Centriflo. Cholate was removed by changing the buffer of the enzyme solution to a 0.1 M potassium phosphate/20% glycerol (v:v)/1 mM EDTA/1 mM DTT/0.1% Emulgen 913 (w:v) solution (pH 7.2) with Amicon Centriflo (Sotokawa et al., 1990). The denatured form, P420, did not exist in the purified wild type and mutants in terms of the absorption spectra of the CO-reduced form.

Phenyl isocyanide was synthesized according to Gokel et al. (1988). Emulgen 913 was obtained from Kao (Tokyo). 7-Ethoxycoumarin was obtained from Aldrich (Milwaukee, WI). Other chemicals of the highest guaranteed grade were purchased from Wako Pure Chemicals (Osaka) and used without further purification.

A stock solution of 7-ethoxycoumarin (50 mM) was made in methanol. It was found in control experiments that methanol up to 5% (v:v) does not affect ligand binding of $P450_d$. Purified phenyl isocyanide was kept at −83 °C. A fresh 43.1 mM stock solution in the buffer was made just before each experiment.

For the spectral experiments, 0.6–0.9 μM $P450_d$ solutions in 1-cm cells were used in the buffer described above. Absorption spectra were recorded on a Shimadzu UV-2200 spectrophotometer equipped with temperature-controlled (Advantec Thermocool LCH-4) cell holder after the completion of temperature adjustment for 5–7 min. The reduction of phenyl isocyanide–$P450_d$ complexes was made by the addition of a slight excess of sodium dithionite.

The contents of the 433-nm peak and 451-nm peak forms (c_{433}, c_{451}) in reduced phenyl isocyanide–$P450_d$ complexes were calculated according to

$$A_{433} = \epsilon^{433}_{433}c_{433} + \epsilon^{451}_{433}c_{451} + c_1 \quad (1)$$

$$A_{451} = \epsilon^{451}_{451}c_{451} + \epsilon^{433}_{451}c_{433} + c_2 \quad (2)$$

where A_n is the absorbance of the complex at n nm, ϵ^m_n is an extinction coefficient of the m-nm form at n nm, and c_1 and c_2 are constant absorptivities at 433 and 451 nm, respectively. These coefficients were experimentally obtained and did not differ between the solutions in the presence and absence of 7-ethoxycoumarin.

Absorbance changes in ligand binding experiments and concentration changes in kinetic experiments were fitted to a set of data points using the nonlinear regression model according to More et al. (1980). Experiments were repeated 2–3 times, and averaged fitting parameters are described. An Arrhenius relation fitting was performed by using the least-squares method according to Draper and Smith (1981). The calculations were made on an NEC PC-9801 personal computer.

Results

Binding of Phenyl Isocyanide with Oxidized $P450_d$. Purified wild-type $P450_d$ is the high-spin complex with the Soret absorption peak at 393 nm. On the addition of phenyl isocyanide to the solution, the Soret peak moved from 393 nm to 430 nm (Figures 1 and 2), which is attributed to the spin change from the high spin to the low spin caused by the addition of the ligand (Sato & Omura, 1978; White & Coon, 1982). These spectral changes depend on the concentration of added ligand according to the Michaelis–Menten-type complex formation (inset of Figure 2). The high-spin Glu318Asp mutant showed the same spectral change with the addition of the ligand. The Glu318Ala mutant was purified as the oxygen-bound low-spin complex with the Soret peak at 416 nm containing 10–15% high-spin complex (Figures 3 and 4) (Shimizu et al., 1991a). The phenyl isocyanide bound form of the mutant Glu318Ala was the carbon-bound complex (White & Coon, 1982). Addition of a large excess of the ligand to the mutants did not make the shoulder around 416 nm totally disappear. The binding parameters (K_s, spectral dissociation constant, and ΔA_{max}, amplitude of maximal spectral changes) derived from the spectral titration study in both the presence and absence of 7-ethoxycoumarin) are listed in Table I. It was found that 7-ethoxycoumarin enhanced the affinity of phenyl isocyanide for the wild type while it decreased the affinity of the ligand for the two mutants. ΔA_{max} values were slightly higher in the presence of 7-ethoxycoumarin

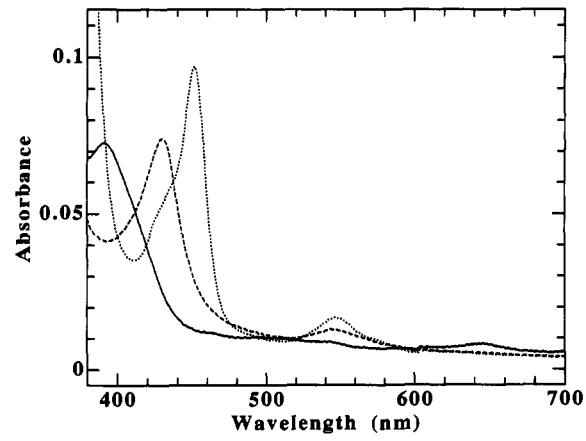

FIGURE 1: Absorption spectra of the ferric (—), phenyl isocyanide (5.48 mM) bound ferric (---), and phenyl isocyanide (5.48 mM) bound ferrous (···) wild-type $P450_d$ (0.72 μM) in the presence of 0.83 mM 7-ethoxycoumarin. The concentration of 7-ethoxycoumarin is nearly at the saturated concentration and is sufficient to make the substrate-bound form from K_m values of 0.1–0.4 mM for all $P450_d$s under study (unpublished results). Spectra were obtained at 18 °C in the buffer (pH 7.2) consisting of 0.1 M potassium phosphate, 20% glycerol (v:v), 1 mM EDTA, and 0.1% Emulgen 913 (w:v).

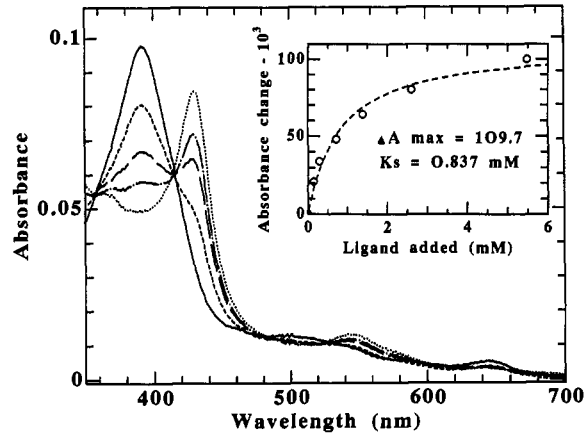

FIGURE 2: Spectral changes of the wild-type P-450_d (0.9 μM) by the addition of 0 (—), 0.14 (---), 0.72 (– –), 2.61 (–·–), and 5.48 (···) mM phenyl isocyanide at 18 °C. Lines in the presence of 0.28 and 1.39 mM are omitted to clarify presentation. Inset shows experimental points and simulated curve on the assumption $\Delta A = \Delta A_{max}[L]/([L] + K_s)$, where [L] is the ligand concentration, ΔA is the absorbance change, ΔA_{max} and K_s are parameters of binding. The buffer used was the same as that in Figure 1.

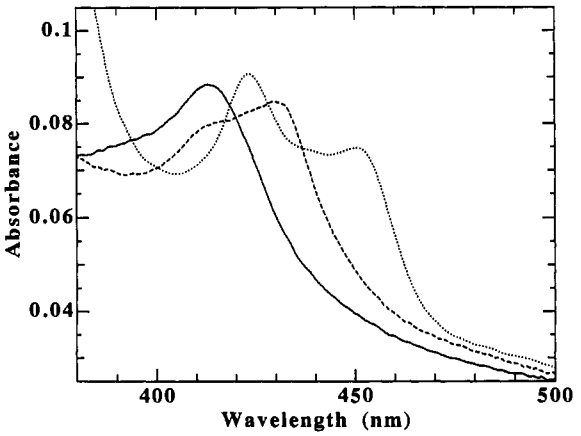

FIGURE 3: Soret absorption spectra of the ferric Glu318Ala mutant (0.66 μM)(—), phenyl isocyanide (5.48 mM) bound ferric mutant (---), and phenyl isocyanide (5.48 mM) bound ferrous mutant (···) at 17 °C. The buffer used was the same as that in Figure 1.

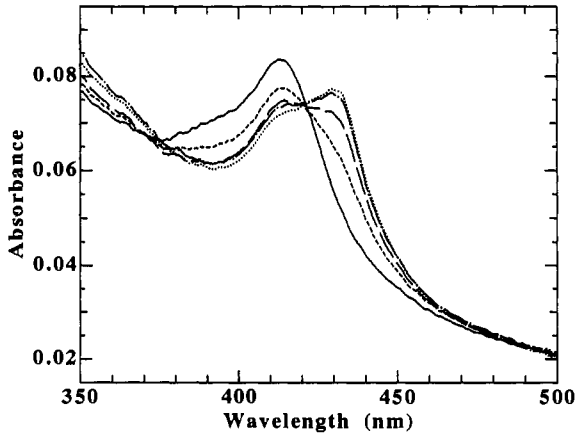

FIGURE 4: Soret spectral changes of the ferric Glu318Ala mutant (0.66 μM) by the addition of phenyl isocyanide at 17 °C. The line assignments are the same as those in Figure 2. The buffer used was the same as that in Figure 1.

for all three enzyme preparations compared with those obtained in its absence.

Table I: Optical Absorption Spectra and Parameters of Phenyl Isocyanide Binding to Oxidized Wild-Type and Mutant $P450_d$s

sample	Soret maxima (nm) –PheNC[a]	Soret maxima (nm) +PheNC[a]	$\Delta A_{max} \times 10^3$ (optical units)[b]	K_s[c] (μM)
wild type	393	430	101 ± 9	741 ± 96
+0.83 mM 7-EC[d]	393	430	110 ± 2	438 ± 68
Glu318Asp	393	430	36 ± 4	385 ± 16
+0.83 mM 7-EC[d]	393	430	47 ± 1	1280 ● 111
Glu318Ala	416	430	33 ± 1	190 ± 14
+0.83 mM 7-EC[d]	416	430	50 ± 7	394 ± 22

[a] PheNC, phenyl isocyanide. [b] Since we did not obtain perfect low-spin PheNC complexes for the mutants due to denaturation by the addition of large excess PheNC, we tentatively describe ΔA_{max} instead of extinction coefficients. ΔA_{max} and K_s values were used to fit experimental values to the equation in the legend of Figure 2. [c] Data are means of 2–3 experiments ± SE. [d] 7-EC, 7-ethoxycoumarin.

Reduced $P450_d$–Phenyl Isocyanide Complexes. Reduction of $P450_d$–phenyl isocyanide complexes by sodium dithionite caused remarkable spectral changes (Figures 1 and 3). Both the wild type and the mutants had a Soret peak at 451 nm and a visible peak at 550 nm. However, both mutants had an additional Soret peak at 423 nm. No considerable spectral difference was observed between the solutions in the presence and absence of 7-ethoxycoumarin.

Keeping reduced $P450_d$–phenyl isocyanide solutions at an ambient temperature led to a decrease of the peak at 451 nm with a concomitant increase of a new peak at 433 nm (Figure 5). Note that the mutants Glu318Asp and Glu318Ala had a constant absorbance at 423 nm (Figure 5B,C), which did not change during the time-dependent measurements.

The spectral transition from the peak at 451 nm to the peak at 433 nm corresponded to the first-order kinetics (Figure 6). The temperature dependence of the conversion kinetics of the reduced wild type–phenyl isocyanide complex was studied in the temperature range from 25 to 36 °C in both the presence and absence of 7-ethoxycoumarin. Mutant complexes exhibited thermal unstability, and thus, the same rates of the conversion were observed from 16 to 25 °C. By changing the

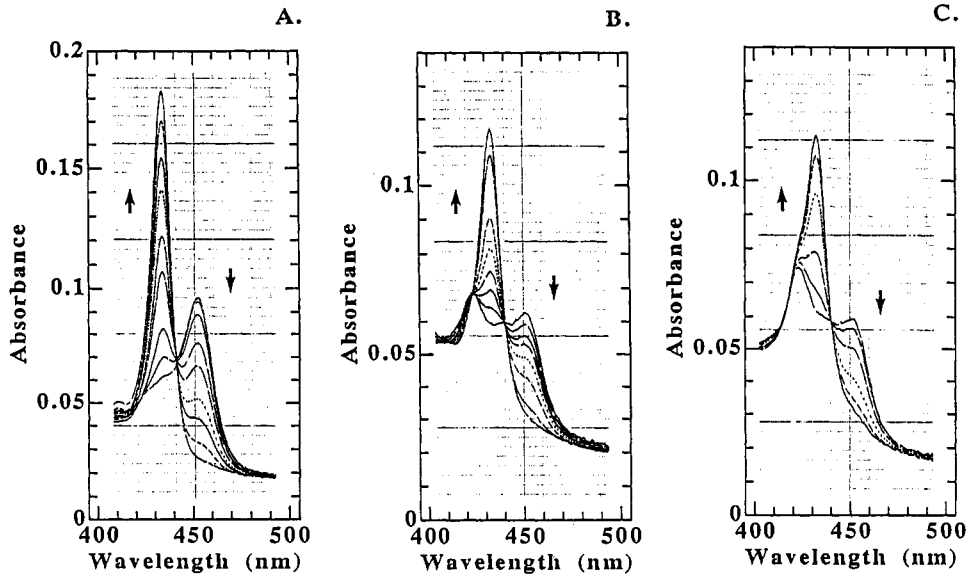

FIGURE 5: Soret spectral changes from the 451-nm peak to the 433-nm peak for the ferrous–phenyl isocyanide (0.43 mM) complexes for the wild-type P-450_d (0.72 μM) (A), the Glu318Asp mutant (0.59 μM) (B), and the Glu318Ala mutant (0.66 μM) (C). Spectra were obtained after 1, 5, 20, 60, 90, 143, 200, 310, and "∞" min at 30 °C for the wild type; after 1, 20, 35, 55, 75, 140, 300, and "∞" min at 17 °C for the mutant Glu318Asp and after 1, 15, 30, 90, 215, and "∞" min at 17 °C for the mutant Glu318Ala. The "∞" minute points were obtained after heating of the sample up to 50 °C. Buffer used was the same as that in Figure 1.

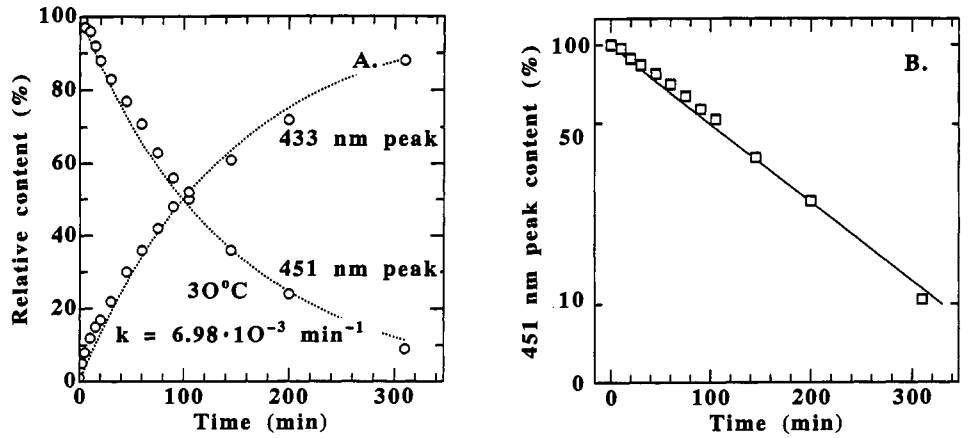

FIGURE 6: Relative changes (A) of the peaks at 451 nm and at 433 nm for phenyl isocyanide–ferrous wild-type complex at 30 °C. Experimental points (O) were obtained from the peak heights in Figure 5A according to eqs 1 and 2 under Experimental Procedures. They were fitted to a straight line in semilog plots (B) to obtain reaction rate constants. Dotted lines in (A) were calculated from first-order kinetics $A = (100\%)\exp(-kt)$ and $B = (100\%)(1 - \exp(-kt))$, where A and B are the contents of the 451-nm and 433-nm peak forms, respectively, and k is the first-order kinetic constant derived from the fitting.

Table II: Kinetic Parameters of the Conversion Reaction of Reduced $P450_d$–Phenyl Isocyanide Complexes[a]

sample	ln k_0	E_{act} (kcal/mol)	r	k^b (min^{-1})
wild type	109.6 ± 9.4	68.7 ± 5.7	0.987	1.07×10^{-3}
+0.83 mM 7-EC[c]	171.9 ± 10.5	106.8 ± 6.4	0.993	1.09×10^{-4}
Glu318Asp	81.2 ± 15.0	49.6 ± 8.7	0.957	5.68×10^{-2}
+0.83 mM 7-EC[c]	60.9 ± 8.8	37.9 ± 5.1	0.974	3.55×10^{-2}
Glu318Ala	59.5 ± 4.2	36.8 ± 2.4	0.992	5.65×10^{-2}
+0.83 mM 7-EC[c]	68.1 ± 5.2	42.6 ± 3.0	0.990	1.65×10^{-2}

[a] Data (±SE) were obtained by least-squares fitting of 5–6 points to a linearized Arrhenius equation, $\ln k = \ln k_0 - E_{act}/RT$, where k_0 is the preexponential factor and E_{act} is the activation energy. The correlation coefficient (r) is also shown for reference. [b] First-order kinetic constant at 24 °C calculated from the obtained parameters ln k_0 and E_{act}. [c] 7-EC, 7-ethoxycoumarin.

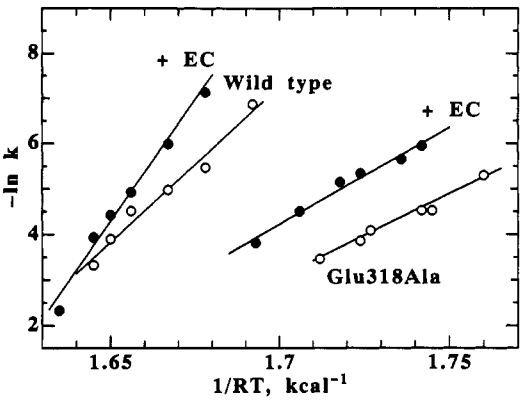

FIGURE 7: Conversion kinetic constants as a function of temperature in the presence (●) and absence (O) of 7-ethoxycoumarin for the wild type and Glu318Ala mutant.

temperature of the complex solutions, Arrhenius plots (ln k vs $1/RT$) were obtained (Figure 7) (Table II). Calculated k values reflect the thermal stability of the protein molecules (Table II). The values of kinetic constants (0.0001–0.1 min^{-1}) and activation energies (40–100 kcal/mol) observed in this study are in good agreement with those observed for general protein folding (Beasty et al., 1986; Pace et al., 1989; Jaenicke, 1991).

Considerable differences in the activation energy of these conversion reactions were observed among the wild-type and mutant proteins in the presence and absence of 7-ethoxycoumarin. For example, E_{act} = 68.7 kcal/mol for the wild type was higher than those for the mutants. In addition, in the presence of 7-ethoxycoumarin the activation energy for the wild type is higher than that in the absence of the substrate by about 40 kcal/mol. In contrast, 7-ethoxycoumarin did not practically influence the activation energy of the mutants. These findings may reflect the properties of protein core packing for the wild-type and the mutant proteins of $P450_d$.

Discussion

The results presented here, together with previous spectral studies (Shimizu et al., 1991a,b) demonstrate the important role of the putative distal region of the heme of P450 in the $P450_d$–ligand interactions. There may now be little doubt that the region at least from the Glu318 to Thr322 residues is located at the distal site of the heme in $P450_d$ and might correspond to the central part of the distal I-helix of $P450_{cam}$ (Poulos et al., 1985, 1987).

Glu318 at the putative distal site of $P450_d$, which corresponds to Asp251 in $P450_{cam}$, is highly conserved for all P450s as either Glu or Asp (Nelson & Strobel, 1988, 1989; Gotoh & Fujii-Kuriyama, 1989). From the X-ray crystal structure of $P450_{cam}$, Asp251 seems to stabilize the structure of the distal site of the heme by forming an ion pair with Arg186 or Lys178 of the F-helix (Poulos et al., 1987). Asp251 of $P450_{cam}$ is also involved in the complex formation with 2-phenylimidazole, preventing direct coordination of nitrogen of this molecule to the heme iron (Poulos & Howard, 1987). This kind of interaction was confirmed for $P450_d$ by a site-directed mutagenesis study (Shimizu et al., 1991a). Namely, the imidazole nitrogen atom of 2-phenylimidazole appeared to directly coordinate to the heme of the mutant Glu318Asp of $P450_d$, in contrast to the wild type where the imidazole nitrogen will interact with the heme in an indirect way as observed for $P450_{cam}$.

It is known that mammalian P450s isolated from microsomal membranes associate themselves in the aqueous solution like other integral membrane proteins (Sato & Omura, 1978; Golly et al., 1988). P450 exists in the monomeric state at a concentration of Emulgen 913 more than 0.026% (w:v) (Bachmanova et al., 1989; Hildebrandt et al., 1989a; Krainev et al., 1991).

Interaction of Phenyl Isocyanide with Oxidized $P450_d$s. Bindings of phenyl isocyanide to the wild type and mutant of $P450_d$ formed low-spin complexes (Figures 1–4) as previously observed for other P450s (Ichikawa & Yamano, 1968; Tsubaki et al., 1989). The Glu318Asp– and Glu318Ala–phenyl isocyanide complexes had a shoulder around 416 nm in their absorption spectra in contrast to the wild type. An addition

of a large excess of phenyl isocyanide made this shoulder disappear. However, for the mutant proteins, phenyl isocyanide does not make a perfect low-spin complex without destroying the heme environment leading to P420.

The addition of 7-ethoxycoumarin enhanced the binding of phenyl isocyanide to the wild-type $P450_d$ (Table I). This is in contrast the results observed for mutant complexes, in which 7-ethoxycoumarin decreased the affinity of phenyl isocyanide to the heme. These findings suggest that there are differences in the structure of the heme environment between the wild type and the mutants.

Recent X-ray crystal studies on CO- and phenyl-heme iron complexes of $P450_{cam}$ (Raag & Poulos, 1989, 1991) show that these ligand molecules do not provide appreciable steric hindrance on the substrate binding pocket but may produce large changes in a proposed substrate access channel region of the hemoprotein. It was proposed that the space of the distal site in the wild-type $P450_d$ is more narrow or tight in contrast to that in other P450s (Sotokawa et al., 1990). The 7-ethoxycoumarin molecule is a relatively large molecule, and its binding to the wild-type $P450_d$ may induce rearrangements in the distal site/substrate access channel, probably by making this region slightly broader. This structural change may cause the enhancement of phenyl isocyanide binding to the wild type. On the other hand, it can be speculated for the mutant proteins that 7-ethoxycoumarin binds to a site that is newly created near the heme in a structurally less packed form. It may interfere with the binding of phenyl isocyanide to the heme in the presence of 7-ethoxycoumarin.

Reduced $P450_d$-Phenyl Isocyanide Complexes. The absorption spectrum of the reduced wild type-phenyl isocyanide complex was different from those of the reduced mutant complexes (Figures 1-5). Thus, it is suggested again that the mutations at Glu318 largely influenced the structure of the ligand binding site and/or the distal environment of $P450_d$.

Intensities of two Soret peaks of microsomal reduced P450-ethyl isocyanide complexes can be reversed by changing the pH (Imai & Sato, 1966). A similar Soret spectral feature of the purified reduced cytochrome $P450_{scc}$-phenyl isocyanide complex was influenced by adrenodoxin and the temperature (Tsubaki et al., 1989). We have observed in the present study the existence of two conformationally different states in terms of absorption spectra of reduced $P450_d$-phenyl isocyanide complexes, corresponding to the 451-nm peak and the 433-nm peak (Figure 5). From the previously reported spectra of microsomal reduced P450- and P420-ethyl isocyanide complexes (Omura & Sato, 1962; Imai & Sato, 1966), spectral conversions observed here might be correlated with a conversion from $P450_d$ to P420.

Under conditions employed, the conversion process occurred quantitatively and followed first-order kinetics (Figure 6). Note that we used the concentration of phenyl isocyanide up to 0.43 mM ($\sim K_s$) because P450 is readily converted to the inactive form P420 in the presence of excess phenyl isocyanide (Ichikawa & Yamano, 1968).

The Glu318Asp and Glu318Ala mutants exhibited less thermal stability in contrast to that of the wild type (Table II). Thus, rates for the mutants' conversions were observed in the temperature range from 16 to 25 °C while those for the wild type were observed at the 25-36 °C range (Figure 7, Table II). The addition of 7-ethoxycoumarin only slightly influenced the rates of conversion.

More remarkable differences among the wild type and the mutants were observed in the activation energy of these conversion reactions. The activation energy (E_{act} = 68.7 kcal/mol) for the wild type was higher that those (49.6 kcal/mol for Glu318Asp, 36.8 kcal/mol for Glu318Ala) for the mutants. This finding appears to be related to the less rigid packing of mutant's protein core. Namely, the ionic interaction of Asp251-Lys178(Arg186) stabilizes the distal structure in $P450_{cam}$ (Poulos et al., 1987). Lys178 at the end of the F-helix in $P450_{cam}$ is highly conserved at Lys/Arg (Arg242 in $P450_d$) for all P450s (Nelson & Strobel, 1988, 1989; Gotoh & Fujii-Kurijama, 1989). Thus, the Arg242 residue in $P450_d$ may participate in stabilizing an ion pair formation(s). Changes in the length and charge of Glu318 amino acid side chains may destabilize the protein structure of the heme environment in $P450_d$ by altering the ion pair formation. By the Glu → Asp substitution, the amino acid side chain becomes shorter and may impede the ion pair formation. For the Glu318Ala mutation, the formation of the ion pair must be deleted, thus leading to the most nonrigid structure in terms of the activation energy.

7-Ethoxycoumarin affected the activation energy of the conversion for the wild type more remarkably than those of the mutants (Table II). The substrate binding may cause a more stable structure of $P450_d$. This finding is in agreement with the widely accepted idea that the substrate binding to P450 changes the protein structure accompanied with the spin state, redox potential, etc., for $P450_{cam}$, $P450_{lin}$, and $P450_{LM2}$ (Sligar, 1976; Ruckpaul & Rein, 1984; Sligar & Murray, 1986; Hildebrandt et al., 1989b; Hui Bon Hoa et al., 1989).

The free energy change ($\Delta G^* = -5.08$ kcal/mol at 25 °C) due to the substrate binding for the wild type was estimated from the K_m value on the assumption of $K_m = K_d$. It can be served as a part of the increased energy of the intermediate state. The other part of this increment may be attributed to increased rigidity of the core packing of the wild type in the presence of substrate. Very similar results in terms of flexibility were observed for $P450_{cam}$ (Hui Bon Hoa et al., 1989). The remarkable increase of $P450_{cam}$ rigidity in the presence of camphor gives an excess of free energy stabilization of -75.3 kcal/mol.

These features may not be true for the Glu318Asp and Glu318Ala mutants. For the mutants, no considerable effects of the substrate binding on the energy of the intermediate state were observed. This may be explained by taking into consideration the fact that the less rigid heme environment caused by the mutation becomes less sensitive to the binding of the substrate.

Conclusions. It was suggested that Glu318 of $P450_d$ may be one of distal amino acids that play a key role in stabilizing the structure of the distal site of $P450_d$. Some of the findings obtained here were interpreted in terms of protein core packing. It is suggested that the Glu318 → Ala mutation of $P450_d$ may delete an important ionic interaction of Glu318 with Lys or Arg, which is required to keep the thermostable distal structure. Therefore, alterations of the structure of the distal site caused by the Glu318 → Ala mutation may be subsequent effects on the protein molecule. Results obtained here are in good agreement with suggestions derived from X-ray crystal structure (Poulos et al., 1985, 1987) and alignments of amino acid sequences of P450s (Nelson & Strobel, 1988, 1989; Gotoh & Fujii-Kuriyama, 1989) in that the whole protein structure of membrane-bound P450 may be similar to that of bacterial $P450_{cam}$ (Krainev et al., 1991).

These results may contrast with the effect of point mutations at a site that is supposed to be too far from the P-450 active site for another P450 (Lindberg & Negishi, 1989; Iwasaki et al., 1991). Substitutions of Cys at the putative remote site

with polar amino acids caused spin changes from the high-spin state to the low-spin state (Iwasaki et al., 1991). For $P450_d$, in contrast, the substitution of Glu318 with Ala at the putative distal site caused the spin change from the high-spin state to the low-spin state. We can again imply that mutations affected the active site structure of $P450_d$ through the changes in the tertiary structure.

ACKNOWLEDGMENTS

The highly qualified assistance of Dr. Hideo Konami in computer applications is gratefully acknowledged.

Registry No. Glu, 56-86-0; heme, 14875-96-8; iron, 7439-89-6; 7-ethoxycoumarin, 31005-02-4.

REFERENCES

Bachmanova, G. I., Kanaeva, I. P., Kondrashin, S. K., Skotselyas, E. D., Kuznetsova, G. P., & Archakov, A. I. (1989) in *Cytochrome P-450: Biochemistry and Biophysics* (Schuster, I., Ed.) pp 300–303, Taylor & Francis, London.

Beasty, A. M., Hurle, M. R., Manz, J. T., Stackhouse, T., Onuffer, J. J., & Matthews, C. R. (1986) *Biochemistry 25*, 2965–2974.

Draper, N. R., & Smith, H. (1981) *Applied Regression Analysis*, 2nd ed., John Wiley & Sons, New York.

Furuya, H., Shimizu, T., Hirano, K., Hatano, M., Fujii-Kuriyama, Y., Raag, R., & Poulos, T. L. (1989) *Biochemistry 28*, 6848–6857.

Gokel, G. W., Widera, R. P., & Weber, W. P. (1988) *Org. Synth. Coll.* 6, 232–235.

Golly, I., Hlavica, P., & Schartau, W. (1988) *Arch. Biochem. Biophys. 260*, 232–240.

Gotoh, O., & Fujii-Kuriyama, Y. (1989) in *Frontiers in Biotransformation* (Ruckpaul, K., & Rein, H., Eds.) Vol. I, pp 195–234, Akademie-Verlag, Berlin, and Taylor & Francis, London.

Hildebrandt, P., Garda, H., Stier, A., Bachmanova, G. I., Kanaeva, I. P., & Archakov, A. I. (1989a) *Eur. J. Biochem. 186*, 383–388.

Hildebrandt, P., Greinert, R., Stier, A., & Taniguchi, H. (1989b) *Eur. J. Biochem. 186*, 291–302.

Hui Bon Hoa, G., DiPrimo, C., Sligar, S. G., & Douzou, P. (1989) in *Cytochrome P-450: Biochemistry and Biophysics* (Schuster, I., Ed.) pp 269–271, Taylor & Francis, London.

Ichikawa, Y., & Yamano, T. (1968) *Biochim. Biophys. Acta 153*, 753–765.

Imai, Y., & Sato, R. (1966) *Biochem. Biophys. Res. Commun. 23*, 5–11.

Imai, Y., & Sato, R. (1968) *J. Biochem. 63*, 270–273.

Iwasaki, M., Juvonen, R., Lindberg, R., & Negishi, M. (1991) *J. Biol. Chem. 266*, 3380–3382.

Jaenicke, R. (1991) *Biochemistry 30*, 3147–3161.

Krainev, A. G., Weiner, L. M., Kondrashin, S. K., Kanaeva, I. P., & Bachmanova, G. I. (1991) *Arch. Biochem. Biophys. 288*, 17–21.

Lindberg, R. L., & Negishi, M. (1989) *Nature 339*, 632–634.

Mims, M. P., Porras, A. G., Olson, J. S., Noble, R. W., & Peterson, J. A. (1983) *J. Biol. Chem. 258*, 14219–14232.

More, J. J., Garbow, B. S., & Hillstrom, K. E. (1980) User Guide for MINIPACK-1, Argonne National Labs Report ANL-80-74, Argonne.

Nebert, D. W., & Gonzalez, F. G. (1987) *Annu. Rev. Biochem. 56*, 945–993.

Nelson, D. R., & Strobel, H. W. (1988) *J. Biol. Chem. 263*, 6038–6050.

Nelson, D. R., & Strobel, H. W. (1989) *Biochemistry 28*, 656–660.

Omura, T., & Sato, R. (1962) *J. Biol. Chem. 237*, PC1375–1376.

Pace, C. N., Shirley, B. A., & Thomson, J. A. (1989) in *Protein Structure: A Practical Approach* (Creighton, T. E., Ed.) pp 311–330, IRL Press, Oxford.

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. (1987) *J. Mol. Biol. 195*, 687–700.

Raag, R., & Poulos, T. L. (1989) *Biochemistry 28*, 7586–7592.

Raag, R., & Poulos, T. L. (1991) *Biochemistry 30*, 2674–2684.

Ruckpaul, K., & Rein, H., Eds. (1984) *Cytochrome P-450*, Akademie-Verlag, Berlin.

Sato, R., & Omura, T., Eds. (1978) *Cytochrome P-450*, Kodansha, Tokyo, and Academic Press, New York.

Shimizu, T., Hirano, K., Takahashi, M., Hatano, M., & Fujii-Kuriyama, Y. (1988) *Biochemistry 27*, 4138–4141.

Shimizu, T., Sadeque, A. J. M., Sadeque, G. N., Hatano, M., & Fujii-Kuriyama, Y. (1991a) *Biochemistry 30*, 1490–1496.

Shimizu, T., Ito, O., Hatano, M., & Fujii-Kuriyama, Y. (1991b) *Biochemistry 30*, 4659–4662.

Sligar, S. G. (1976) *Biochemistry 15*, 5399–5406.

Sligar, S. G., & Murray, R. I. (1986) in *Cytochrome P-450, Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 429–503, Plenum Press, New York.

Sotokawa, H., Shimizu, T., Furuya, H., Sadeque, A. J. M., Hatano, M., Ohba, Y., Iwaizumi, M., & Fujii-Kuriyama, Y. (1990) *Biochim. Biophys. Acta 1037*, 122–128.

Tsubaki, M., Hiwatashi, A., & Ichikawa, Y. (1989) *Biochemistry 28*, 9777–9784.

White, R. E., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 3073–3083.