Site-Directed Mutageneses of Rat Liver Cytochrome P-450$_d$: Axial Ligand and Heme Incorporation†

Toru Shimizu,*,‡ Kenji Hirano,‡ Masae Takahashi,‡ Masahiro Hatano,*,‡ and Yoshiaki Fujii-Kuriyama§,‖

Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Katahira, Sendai 980, Japan, and Department of Biochemistry, Cancer Institute, Japanese Foundation of Cancer Research, Toshima-ku, Tokyo 170, Japan

Received October 7, 1987; Revised Manuscript Received January 27, 1988

ABSTRACT: By oligonucleotide-directed mutageneses, 13 substitutions of amino acids at the carboxy-terminal region of rat liver cytochrome P-450$_d$ were done as follows: (A) Phe-449 → Tyr; (B) Gly-450 → Ser; (C) Leu-451 → Ser; (D) Gly-452 → Glu; (E) Lys-453 → Glu; (F) Arg-454 → Leu; (G) Arg-455 → Gly; (H) Cys-456 → Tyr; (I) Cys-456 → His; (J) Ile-457 → Ser; (K) Gly-458 → Glu; (L) Glu-459 → Ala; (M) Ile-460 → Ser. The CO-bound reduced forms of the wild type and mutants B–G, J, L, and M gave Soret peaks at 448 nm. The CO complex of mutant A gave a Soret peak at 445 nm. The intensities of the CO-bound forms of mutants A, C, D, and J were very small compared with that of the wild-type complex. The CO-reduced forms of mutants H, I, and K did not give a Soret peak around 450 nm at all. The 448-nm peak of mutant F was unstable and quickly disappeared with the concomitant appearance of a peak at 420 nm. These findings, together with data in the literature, indicate that (1) invariant Cys-456 at the carboxy-terminal region of P-450$_d$ is an axial ligand of the heme iron of the eukaryotic P-450's; (2) hydrophobic amino acids such as Phe-449, Leu-451, Gly-452, and other hydrophobic amino acids such as Ile-457 and Gly-458 next to the axial ligand are apparently very important for the apoprotein to hold and/or incorporate the heme plane at the active site of P-450; and (3) Arg-454 interacts with the heme propionate as His-355 of P-450$_{cam}$ does [Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., & Kraut, J. (1985) *J. Biol. Chem. 260*, 16122–16130].

Cytochrome P-450 (P-450)[1] is a family of heme-containing monoxygenases (Sato & Omura, 1978; Ortiz de Montellano, 1986). In contrast to other hemoproteins such as metmyoglobin (metMb) or methemoglobin (metHb), it has been suggested that P-450 has an axial ligand of cysteine, which causes many abnormal spectroscopic properties of P-450's and is related to hydroxylation activities of P-450's (Sato & Omura, 1978; Ortiz de Montellano, 1986). Gotoh et al. (1983) pointed out from comparison of primary structures of P-450$_d$ and P-450$_{cam}$ that there are two important cysteine residues in P-450's, one of which is situated at the amino-terminal region (HR1 region) and another of which is invariant and is situated at the carboxy-terminal region (HR2 region) (Figure 1). Black et al. (1982) and Haniu et al. (1984) initially claimed that the cysteine residue in the HR1 region of liver microsomal P-450's was the axial ligand and was responsible for the abnormal spectral properties of eukaryotic P-450's. Further comparison of additional amino acid sequences of P-450's revealed that a cysteine is strictly conserved in the HR2, but not in the HR1, and, therefore, suggested that the cysteine in the HR2 is the fifth ligand of the heme iron (Morohashi et al., 1984; Kawajiri et al., 1984). A recent crystallographic study of water-soluble bacterial P-450$_{cam}$ showed that a cysteine residue in the HR2 region of P-450$_{cam}$ is the fifth ligand to the heme iron of the protein (Poulos et al., 1985, 1986). Eukaryotic P-450's are membrane-bound proteins and are very different from bacterial P-450$_{cam}$ in many respects (Sato & Omura, 1978; Ortiz de Montellano, 1986).

P-450$_d$ is induced in rat liver microsomes by isosafrole and has an efficient catalytic activity toward 2-hydroxylation of 17β-estradiol (Ryan et al., 1980). This activity is in contrast with those of other forms of rat liver P-450's induced by phenobarbital and 3-methylcholanthrene. Recently, P-450$_d$ was abundantly expressed in the yeast *Saccharomyces cerevisiae* (Shimizu et al., 1986) with P-450$_d$ cDNA (Kawajiri et al., 1984) and acid phosphatase promoter (Miyanohara et al., 1983). The expression efficiency was more than 1% of the total yeast protein. The catalytic activity and the absorption intensity of the expressed P-450$_d$ were satisfactorily identical with those of the microsomal P-450, which allowed us to do the protein engineering of P-450$_d$ to study the structure–function relationship of P-450. We describe here from the site-directed mutagenesis study that (1) the Cys-456 in the HR2 region of membrane-bound eukaryotic P-450$_d$ is the axial ligand of the heme iron and (2) conserved hydrophobic amino acids near the heme binding site are essential for holding and/or incorporation of the heme in the active site. It seemed very likely that the heme environment of eukaryotic P-450$_d$ is very similar to that of P-450$_{cam}$ derived from crystallographic study (Poulos et al., 1985, 1986).

Experimental Procedures

An expression vector consisting of P-450$_d$ cDNA and acid phosphatase promoter was constructed as described (Shimizu

† This work was supported in part by Grants-in-Aid for Special Project Research (61130001, 62120001) from the Ministry of Education, Science and Culture of Japan to M.H.

‡ Tohoku University.

§ Cancer Institute.

‖ Present address: Department of Chemistry, Faculty of Science, Tohoku University, Aoba, Sendai 980, Japan.

[1] Abbreviations: P-450, cytochrome P-450; P-450$_d$, cytochrome P-450$_d$; HR1 region, highly homologous amino-terminal region; HR2, highly homologous carboxy-terminal region; P-450$_{cam}$, cytochrome P-450 purified from camphor-grown *Pseudomonas putida*; isosafrole, 5-(1-propenyl)-1,3-benzodioxole; 17β-estradiol, estra-1,3,5(10)-triene-3,17β-diol; phenobarbital, 5-ethyl-5-phenyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione; 3-methylcholanthrene, 1,2-dihydro-3-methylbenz[*j*]aceanthrylene; HPLC, high-performance liquid chromatography; ESR, electron spin resonance.

0006-2960/88/0427-4138$01.50/0

F	G	L	G	K	R	R	C456	I	G	E	I				rat P-450$_d$
F	G	L	G	K	R	R	C		I	G	E	I			mouse P-450$_3$
F	G	L	G	K	R	K	C		I	G	E	T			rat P-450$_c$
F	G	L	G	K	R	K	C		I	G	E	T			mouse P-450$_1$
F	S	T	G	K	R	I	C		L	G	E	G			rat P-450$_b$
F	S	T	G	K	R	I	C		L	G	E	G			rat P-450$_e$
F	G	L	G	K	R	R	C		I	G	E	T			rabbit P-450$_{LM4}$
F	S	L	G	K	R	I	C		L	G	E	G			rabbit P-450$_{LM2}$
F	S	A	G	K	R	A	C		V	G	E	G			rabbit P-450$_{LM3b}$
F	G	W	G	V	R	Q	C		V	G	R	R			bovine P-450$_{scc}$
F	G	H	G	S	H	L	C		L	G	Q	H			P. putida P-450$_{cam}$

FIGURE 1: Amino acid sequences of P-450's in the HR2 region (Gotoh et al., 1983; Black & Coon, 1986). 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.

et al., 1986). Host cells, *Saccharomyces cerevisiae* AH22 (*a leu2 his4 can1 cir*$^+$), and its ATPase constitute derivatives, AH22pho80, were cultured as described (Miyanohara et al., 1983; Shimizu et al., 1986).

Site-directed mutageneses were done as previously described (Zoller & Smith, 1983; Taylor et al., 1985a,b). We used 21-mer primers for 13 mutageneses in phage M13. Thirteen oligonucleotides were synthesized by the Gene Assembler (Pharmacia) and were subsequently purified by HPLC equipped with a Mono Q column (Pharmacia).

Reagents used were of the highest guaranteed grade and were used without further purification. Restriction enzymes and DNA modifying enzymes were purchased from Takara Shuzo (Kyoto) and Amersham International (Amersham, U.K.). [α-^{32}P]deoxyCTP and [γ-^{32}P]ATP were purchased from Amersham International (Amersham, U.K.).

Nucleotide sequencing of the mutagenized P-450$_d$ gene was done by the dideoxy chain terminator method with [α-^{32}P]-deoxyCTP (Zoller & Smith, 1983; Sanger et al., 1977) (see paragraph at end of paper regarding supplementary material). For nucleotide sequences of mutant P-450$_d$'s, we picked up five clones (plagues) at random directly from the agar plate of M13 for every mutant (Taylor et al., 1985a,b). In all cases decribed here, more than three clones of five clones which we picked up and sequenced were mutated. Sequencing primer used was 17-mer which corresponds to nucleotides from His-475 to Thr-480. We also strictly checked every mutant for whether a mutation(s) at another position(s) occurred or not.

Catalytic activities against 17β-estradiol were monitored by both absorption change at 340 nm of NADPH and gas chromatography of the trimethylsilyl derivative of the product. For gas chromatography, after 0.5 mM 17β-estradiol was incubated with yeast microsomes containing 1–10 nmol of P-450$_d$, 200 units of cytochrome P-450 reductase, and 1 mM NADPH at 37 °C for 20 min, the product was twice extracted by ethyl acetate and was air-dried. The dried residue was dissolved in 20 μL of pyridine. *N*-(Trimethylsilyl)imidazole (20 μL) was added to the pyridine solution, and the solution was kept at room temperature (20–25 °C) for more than 2 h. The trimethylsilyl derivatives of the substrate and the product were quantitatively formed. The pyridine solution containing the trimethylsilyl derivatives was applied to Yanako Gas Chromatograph Model 80 equipped with a silicon column OV-61 (Gas Chorm. Indus.) (3 mm × 2 m, 270 °C) and flame ionization detector (cf. supplementary material). Activities

Table I: Difference Absorption Spectra of the CO-Bound Forms of Reduced Wild P-450$_d$ and Mutant P-450$_d$'s Expressed in Yeast[a]

	λ_{max} (nm)	intensity at 448 nm[b]	intensity at 420 nm[c]
wild	448	1.0 ± 0.2	<0.05
mutant, A, *Phe-449 → Tyr	445	<0.2	<0.05
mutant B, Gly-450 → Ser	448	1.0 ± 0.2	<0.05
mutant C, Leu-451 → Ser	448	<0.2	<0.05
mutant D, *Gly-452 → Glu	448	<0.2	<0.05
mutant E, Lys-453 → Glu	448	1.0 ± 0.2	<0.05
mutant F, Arg-454 → Leu	448	0.9 ± 0.2	<0.10
mutant G, Arg-455 → Gly	448	1.0 ± 0.2	<0.05
mutant H, *Cys-456 → Tyr			<0.20
mutant I, *Cys-456 → His			<0.20
mutant J, Ile-457 → Ser	448	<0.2	<0.05
mutant K, *Gly-458 → Glu			<0.20
mutant L, Glu-459 → Ala	448	1.0 ± 0.2	<0.05
mutant M, Ile-460 → Ser	448	1.0 ± 0.2	<0.05

[a] (*) Invariant amino acids. [b] Intensities were normalized to that of the wild type, taking the cell number of the cultured yeast into consideration, and were averaged from at least four experiments. For mutant A the intensity was taken at 445 nm. [c] Intensities at 420 nm were compared to that (unity) at 448 nm of the wild type.

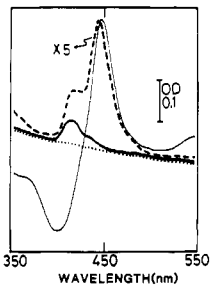

FIGURE 2: Difference absorption spectra of the CO-bound forms of reduced wild P-450$_d$ (—), mutant A (– –) and mutant I (thick line) expressed in the yeast. The yeast cultured in 400 mL of medium was harvested and suspended in 16 mL of 0.1 M potassium phosphate buffer (pH 7.4). The reference cuvette contained the yeast solution with sodium dithionite while the sample cuvette contained the yeast solution with CO gas and sodium dithionite. Spectra were obtained at 20 °C.

were determined under conditions in which the rate of product formation was constant with respect to protein and time of incubation.

Absorption spectra were obtained on a Shimadzu UV365 recording spectrometer equipped with an end-on photomultiplier (HAMAMATSU R-375). ESR spectra were obtained on a Varian E-112 spectrometer equipped with a liquid helium flow temperature controller.

Results

Tweleve amino acids of P-450$_d$ in the HR2 region (Figure 1) (Morohashi et al., 1984; Gotoh et al., 1983; Kawajiri et al., 1984), Phe-449, Gly-450, Leu-451, Gly-452, Lys-453, Arg-454, Arg-455, Cys-456, Ile-457, Gly-458, Glu-459, and Ile-460, were changed to other amino acids by oligonucleotide-directed mutagenesis (Zoller & Smith, 1983; Taylor et al., 1985a,b) as described in Table I. Polarities of amino acids were purposely changed except for the mutations H and I. Cys-456 was substituted by tyrosine (mutant H) or histidine (mutant I). Since P-450$_d$ was abundantly expressed in the yeast, the optical absorption spectrum of the CO-bound form of reduced P-450$_d$ was easily obtainable by measuring the spectra of the yeast in situ. The optical absorption difference spectrum of the CO-bound form of reduced P-450$_d$ expressed in the yeast is shown in Figure 2. The absorption maximum of the CO-bound form of wild P-450$_d$ was located at 448 nm. The CO-

Table II: Catalytic Activities toward 17β-Estradiol of Wild and Mutant P-450$_d$

	activities [nmol min^{-1} (nmol of P-450$_d$)$^{-1}$][a]
rat liver P-450$_d$	14.0[b]
expressed wild P-450$_d$	12.3
mutant B	16.4
mutant E	18.5
mutant F	9.9
mutant G	14.5
mutant L	17.3
mutant M	25.1

[a] Activities were obtained for yeast microsomes by the absorption change at 340 nm of NADPH in the presence of cytochrome P-450 reductase (Ryan et al., 1980; Shimizu et al., 1986). Activities were also checked by gas chromatography for trimethylsilyl derivatives of the product (see supplementary material). For mutants A, C, D, and J, it was not feasible to quantitate catalytic activities because those P-450$_d$ mutants in microsomes were very unstable and the microsomes had only denatured-form cytochrome P-420. Incubations of 17β-estradiol in the microsomes of those mutants together with cytochrome P-450 reductase and NADPH did not give 2-hydroxy-17β-estradiol at all in terms of gas chromatography. The yeast not harboring the P-450$_d$ expression vector did not show catalytic activity toward 17β-estradiol. [b] Ryan et al., 1980.

bound form of reduced mutant A had the Soret absorption peak at 445 nm, while the CO-bound forms of mutants B–G, I, L, and M had the Soret peaks at 448 nm. The CO-bound forms of the reduced mutants H, I, and K did not show any absorption peak around 450 nm at all. The intensities of the Soret absorption peaks of the mutants A, C, D, and J were much smaller than those of the wild and the mutants B, E, F, G, L, and M by less than one-fifth (Table I). From Western blottings, it was decidedly indicated that the mutant A, C, D, H, I, J and K proteins were expressed in the yeast to the same extent as those of the wild type and other mutants.

It was noted here that mutants A, C, D, H, I, J and K did not give a large peak at 420 nm of the free heme in that the intensity of the 420-nm peak was less than 20% of that of the 448-nm peak of the wild type.

The 448-nm peak of mutant F was unstable and quickly disappeared concomitantly with the appearance of a peak at 420 nm (Figure 3). By analogy of the stereo structure of P-450$_{cam}$, Arg-454 is assumed to interact ionically with the propionic acid of the heme.

Catalytic activities of the wild and mutant P-450$_d$'s against 17β-estradiol were obtained (Table II). In most cases, activities of the expressed mutants were nearly equal to that of the wild type or a little higher than that of the wild type.

The expressed wild-type P-450$_d$ had the ESR g_y component at $g = 7.92$ of the high-spin complex at 7 K (not shown). ESR signals ascribed to low-spin heme complexes were not detected in the high magnetic filed region up to 40 K. The ESR g_y for the expressed mutants B, E, F, G, L, and M were essentially the same as that of the wild type.

Discussion

From the studies of site-directed mutagenesis described here (Figure 2), it was indicated that invariant Cys-456 in the HR2 region is the fifth axial ligand of the heme in P-450$_d$ and causes the abnormal Soret absorption peak around 450 nm for the CO-bound form of the reduced hemoprotein.

Lower absorption intensities of the CO-bound forms of the mutants A, C, D, and J may be related to the diminished binding affinities of the heme to the active site of P-450$_d$. When phenylalanine, leucine, and glycine, and isoleucine were substituted by tyrosine, serine, glutamic acid, and serine, respectively, hydrophobic character of the heme environment

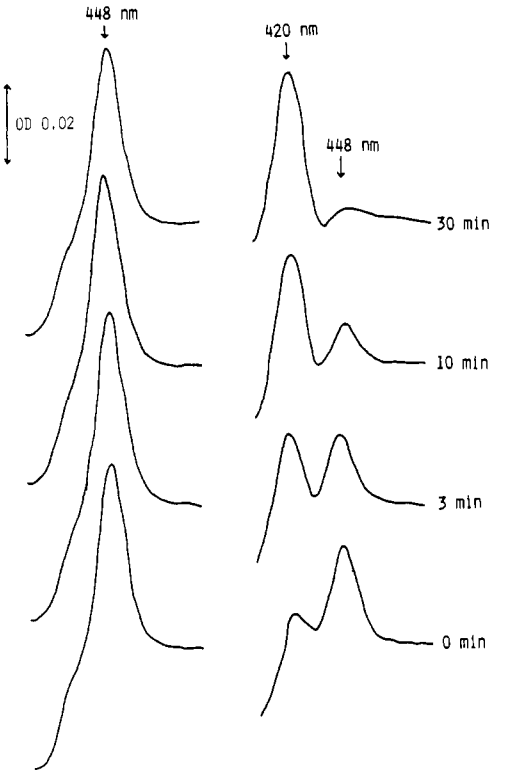

FIGURE 3: Difference spectral changes of the CO-bound form of mutant F. The left column shows the time dependence of the wild type, while the right column shows the time dependence of mutant F. The yeast concentrations of both solutions are not the same. Spectra were obtained at 25 °C.

is reduced together with a slight structural alteration due to the side chains of the amino acids. Generaly, in wild P-450's, the heme plane will be held in the active site of P-450 through both the hydrophobic interaction with their neighboring amino acids and the axial coordination of the internal cysteine to the heme iron. If Ile-457 and Gly-458 participate in the heme binding, then replacing these residues with more hydrophoilic amino acids should alter the heme binding property. The data presented here clearly show this prediction to be the case, thus supporting the view that the cysteine ligand region (HR2) is the same in P-450$_{cam}$ (Poulos et al., 1985, 1986) and in eukaryotic P-450's. Steric and electrostatic repulsion of the glutamic acid residue and/or a conformational change caused by substituting glutamic acid for glycine may also in part reduce the interaction of the heme plane with the apoprotein in mutants D and K.

In mutant F, the heme iron was clearly incorporated into the apoprotein of P-450$_d$ as in the wild type, but the heme iron apparently easily dissociates from the apoprotein in the CO-reduced form (Figure 3). Arg-454 in the wild type was changed to Leu in mutant F. This Arg-454 in P-450$_d$ corresponds to His-355 in P-450$_{cam}$ (Figure 1), which interacts with the heme propionate through ionic interactions (Poulos et al., 1985). Poulos et al. (1985) suggested that in eukaryotic P-450's Arg (Arg-454 for P-450$_d$) may interact with the heme propionates. Our result is in accordance with their suggestion. Since the heme iron was clearly incorporated into the apoprotein of mutant F, Arg-454 may not play an important role for the heme binding in the oxidized form. Arg-454 may rather anchor the reduced heme moiety to the apoprotein of P-450$_d$, since cysteine is difficult to bind to the reduced heme iron and thus the reduced heme iron may be labile in P-450.

The CO-bound forms of the mutants A, C, D, H, I, J, and K should provide the Soret peak at 420 nm of those of the free

CO–heme complex or myoglobin-type CO–heme–imidazole complex. However, the CO-bound mutants A, C, D, H, I, J, and K did not provide a large Soret peak at 420 nm comparable to the 448-nm peak of the wild-$P450_d$, but a very small peak at 420 nm (less than 20% of the 448-nm peak of the wild type) was observed for these complexes. These results suggest that only a small quantity of free heme iron exists in the yeast for the mutants A, C, D, H, I, J, and M. We would speculate that biosynthesis of the heme iron complex in the yeast might be closely associated with the heme binding to the apoprotein of $P\text{-}450_d$. That is, the heme iron might not be synthesized in the yeast when there are not enough receiving sites for the heme. Although degradation of the heme in the yeast was very slow (our unpublished observation), the possibility of this degradation for the low heme concentration in those mutants cannot be ruled out.

It is interesting to note that the substitution of tyrosine for invariant Phe-449 shifted the absorption maximum at 448 nm to 445 nm for the CO-bound reduced $P\text{-}450_d$ (mutant A). P-450's usually have absorption peaks longer than 447 nm for their CO-bound reduced form. It was reported, however, that an absorption maximum of the CO-bound P-450 of a yeast mutant is located at unusually lower wavelengths at 445 nm (King et al., 1985). Interactions of external aromatic compounds with the heme plane in P-450 cause shifts of the absorption maximum of the CO-bound reduced P-450's to lower wavelengths (Horie, 1978).

From ESR findings, it was suggested that once the heme is incorporated into the apoprotein of the mutants B, E, F, G, L, and M, the electronic structures of the high-spin complexes of the mutants may not be different from each other or from that of the wild type. This suggestion was supported by the fact that the catalytic activities of these mutants were not very much different from that of the wild type (Table II). Activities of some mutants were a little higher than that of the wild type except that of mutant F. The mutations around the axial ligand, Cys-456, may influence the electron-transfer process.

In conclusion, this is the first paper which directly tests the role of invariant Cys-456 in the carboxy-terminal region (HR2) as the axial ligand of eukaryotic membrane-bound P-450's. It was also shown that hydrophobic amino acids such as Phe-449, Leu-451, Gly-452, Ile-457, and Gly-458 are important (perhaps prerequisite) for the apoprotein to hold and/or incorporate the heme at the active site of eukaryoatic P-450's. Structural information about the heme environment of water-soluble prokaryotic $P\text{-}450_{cam}$ (Poulos et al., 1985, 1986) seems mostly applicable to the eukaryotic membrane-bound P-450's.

ACKNOWLEDGMENTS

We are very grateful to Dr. K. Sogawa, Cancer Institute, Dr. K. Yutani, Osaka University, Dr. R. Kominami, University of Tokyo, and Dr. K. Morohashi, Kyushu University, for valuable suggestions on techniques of site-directed mutagenesis. We thank Dr. H. Ohba for technical assistance of ESR measurements. We are deeply indebted to Pharmacia (Japan) for giving us access to the Gene Assembler, which facilitated our work very much.

SUPPLEMENTARY MATERIAL AVAILABLE

Nucleotide sequence patterns determined by the dideoxy chain terminator method for the wild-type and mutant $P\text{-}450_d$'s and gas chromatography patterns to identify and quantitate 2-hydroxy-17β-estradiol (7 pages). Ordering information is given on any current masthead page.

REFERENCES

Black, S. D., & Coon, M. J. (1986) in *Cytochrome P-450, Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 161–216, Plenum, New York.

Black, S. D., Tarr, G. E., & Coon, M. L. (1982) *J. Biol. Chem. 257*, 14616–14619.

Gotoh, O., Tagashira, Y., Iizuka, T., & Fujii-Kuriyama, Y. (1983) *J. Biochem. (Tokyo) 93*, 807–817.

Haniu, M., Yuan, P.-M., Ryan, D. E., Levin, W., & Shively, J. E. (1984) *Biochemistry 23*, 2478–2482.

Horie, S. (1978) in *Cytochrome P-450* (Sato, R., & Omura, T., Eds.) pp 73–106, Kodansha, Tokyo, and Academic, New York.

Kawajiri, K., Gotoh, O., Sogawa, K., Tagashira, Y., Muramatsu, M., & Fujii-Kuriyama, Y. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1649–1653.

King, D. J., Wiseman, A., Kelly, D. E., & Kelly, S. L. (1985) *Curr. Genet. 10*, 261–267.

Miyanohara, A., Toh-e, A., Nozaki, C., Hamada, F., Ohtomo, N., & Matsubara, K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1–5.

Morohashi, K., Fujii-Kuriyama, Y., Okada, Y., Sogawa, K., Hirose, T., Inayama, S., & Omura, T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4647–4651.

Ortiz de Montellano, P. R., Ed. (1986) *Cytochrome P-450, Structure, Mechanism and Biochemistry*, Plenum, New York.

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.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

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

Shimizu, T., Sogawa, K., Fujii-Kuriyama, Y., Takahashi, M., Ogoma, Y., & Hatano, M. (1986) *FEBS Lett. 207*, 217–221.

Taylor, J. W., Schmidt, W., Cosstick, R., Okruszek, A., & Eckstein, F. (1985a) *Nucleic Acids Res. 13*, 8749–8764.

Taylor, J. W., Ott, J., & Eckstein, F. (1985b) *Nucleic Acids Res. 13*, 8764–8785.

Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468–500.