Effect of Mutations of Ionic Amino Acids of Cytochrome P450 1A2 on Catalytic Activities toward 7-Ethoxycoumarin and Methanol†

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ABSTRACT: Catalytic efficiencies, percentages of rates of product formation per NADPH oxidized, and rates of product formation per O_2 consumed of ionic mutants of cytochrome P450 1A2 (P450 1A2) were studied. Efficiencies of Lys99Glu, Lys453Glu, and Arg455Glu mutants for the hydroxylation reaction toward 7-ethoxycoumarin in the reconstituted system were much lower than that of the wild type (less than 17%), which corresponds to lower turnover numbers for these mutants. In contrast, the catalytic efficiencies for the hydroxylation reaction toward methanol of the three mutants were more than 45% that of the wild type in spite of these mutants' lower turnover numbers. Turnover numbers and catalytic efficiencies of Arg137Leu and Lys401Glu mutants toward both substrates were comparable to those of the wild type. The electron-transfer rate from the reductase to the heme of P450 1A2 was decreased by 30% upon addition of excess methanol, while it was not influenced by addition of excess 7-ethoxycoumarin. The turnover numbers toward both 7-ethoxycoumarin and methanol as well as the rate constant of electron transfer were decreased by 25–40% by raising the concentration of KCl from 0 to 300 mM in the reconstituted system containing 50 mM potassium phosphate buffer. The turnover numbers toward both substrates of the above-mentioned five ionic mutants caused by *tert*-butyl hydroperoxide in the absence of the reductase and NADPH were comparable to those of the wild type. The effect of phospholipid constituents on the catalytic activity toward 7-ethoxycoumarin of the wild type was also studied. From these findings it is suggested that (1) the electron transfer from the reductase to P450 1A2 and/or the interaction between P450 1A2 and the reductase are modulated by the substrates and thus are different between 7-ethoxycoumarin and methanol systems; (2) Lys401 of P450 1A2 is not directly involved in the interaction with the reductase; (3) the interaction between P450 1A2 and the reductase is partially ionic; and (4) mutations of ionic amino acids such as Lys99, Arg137, Lys401, Lys453, and Arg455 of P450 1A2 do not largely change the heme active site and the substrate-binding site(s).

Interaction of microsomal cytochrome P450 (P450)[1] with NADPH–cytochrome P450 reductase, a flavoprotein, is very important for the electron transfer which is essential in the catalytic function of microsomal P450 (French et al., 1980; Guengerich, 1991; Guengerich et al., 1975; Kanaeva et al., 1992; Müller-Enoch et al., 1984; Oprian et al., 1979; Ortiz de Montellano, 1986; Peterson et al., 1976; Porter & Coon, 1991). It was suggested that the interaction between the two proteins is ionic, involving ionic amino acids such as Lys and Arg for P450 and Asp and Glu for the reductase (Bernhardt et al., 1984, 1988; Nadler & Strobel, 1988, 1991; Shen & Strobel, 1992). In fact, reversing the ionic character of well-conserved ionic amino acids such as Lys94, Lys99, Lys105, Lys440, Lys453, Arg455, and Lys463 of microsomal P450 1A2 by substitution with Glu remarkably decreased the catalytic activity toward 7-ethoxycoumarin and the electron-transfer rate from NADPH and the reductase to the heme of P450 1A2 (Shimizu et al., 1991b). Those important ionic amino acids and/or ionic regions of P450 1A2 apparently correspond with those of $P450_{cam}$ (Davis & Sligar, 1992; Stayton et al., 1989; Stayton & Sligar, 1990) in terms of alignments of amino acid sequences of P450s (Nelson & Strobel, 1988) and the three-dimensional structure of $P450_{cam}$ (Poulos et al., 1985, 1987, 1991). However, a recent report indicated that a charge-pairing is not important in the interaction between a certain P450 and the reductase (Voznesensky & Schenkman, 1992). On the other hand, some hydroxylation reactions are caused by P450 even in the absence of the reductase. For example, hydrophobic hydroperoxides such as cumyl hydroperoxide and *tert*-butyl hydroperoxide are known to cause hydroxylation reactions for P450 in the absence of the reductase and NADPH (Nordblom et al., 1976).

Catalytic activities of microsomal P450s are usually uncoupled from the consumption of O_2 and oxidation of NADPH and are accompanied by the production of H_2O_2 (Guengerich, 1991; Hiroya et al., 1992; Ishigooka et al., 1992; Ortiz de Montellano, 1986). The production of H_2O_2 may make the catalytic reaction of microsomal P450 further complicated since H_2O_2 itself causes hydroxylation reactions (Ortiz de Montellano, 1986; Guengerich, 1991). However, the mechanism of uncoupling of microsomal systems has not been understood in detail. Thus, it seems worthwhile to study in detail the uncoupling mechanism of the above-mentioned ionic mutants of P450 1A2. In addition, use of the hydroperoxide system may be helpful in understanding the role of these ionic amino acids of P450 1A2.

In the present study, we study catalytic efficiencies of mutants at Lys99, Arg137, Lys401, Lys453, and Arg455 of P450 1A2 toward 7-ethoxycoumarin and methanol, taking consideration of the rate of NADPH oxidation, the rate of O_2 consumption, and the rate of H_2O_2 production together with

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[1] Abbreviations: P450, cytochrome P450; P450 1A2, rat liver microsomal cytochrome P450 1A2, CYP1A2, or cytochrome $P450_d$; $P450_{cam}$, cytochrome P450 101, or CYP101 (Gonzalez, 1989; Nebert et al., 1991) purified from *Pseudomonas putida* grown in the presence of camphor; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; 7-ethoxycoumarin, 7-ethoxy-2*H*-1-benzopyran-2-one; phenobarbital, 5-ethyl-5-phenyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione; DLPC, dilauroyl-L-α-phosphatidylcholine.

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turnover numbers. We select 7-ethoxycoumarin and methanol as the substrates because it seemed that the mechanisms of hydroxylation reactions toward both substrates are different from each other (Hiroya et al., 1992; Ishigooka et al., 1992). Note that the Arg137 and Lys401 mutants are purified for the first time and studied in detail in the present study. Rate constants of the electron transfer from the reductase and NADPH to those mutant enzymes are obtained under various conditions. We also obtain catalytic activities toward both substrates of the mutant enzymes caused by *tert*-butyl hydroperoxide in the absence of the reductase and NADPH. The catalytic reactions by *tert*-butyl hydroperoxide are studied in order to evaluate the role of the reductase in the catalytic function and to know how the active sites, the heme-binding site, and the substrate-binding site(s) are influenced by the mutations (Shen & Strobel, 1992). Thus, it is suggested from the catalytic efficiencies of the ionic mutants that the electron-transfer mechanism in 7-ethoxycoumarin hydroxylation may be different from that of methanol hydroxylation. In addition, the interaction between P450 1A2 and the reductase seems ionic to a certain extent. The role of Lys401 of P450 1A2 in the interaction with the reductase is ruled out.

EXPERIMENTAL PROCEDURES

Site-directed mutageneses and expression of P450 1A2 mutants in yeast were carried out as previously described (Shimizu et al., 1991a,b). Approximately 400 nmol (per heme) of P450 1A2 protein was expressed in 1-L yeast culture under optimum conditions. Yeast microsomes were prepared by crushing yeast cells with an MSK cell homogenizer (B. Braun, FRG) and by centrifugation at 120000*g* for 90 min. Solubilization and purification with column chromatographies on ω-amino-*n*-hexyl-Sepharose and hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) were carried out as previously described (Funae & Imaoka, 1987; Shimizu et al., 1991a,b). The wild-type and mutant proteins were purified as a high-spin form with a Soret peak at 393 nm (Shimizu et al., 1991a) and did not contain any denatured form, P420, in terms of the Soret absorption spectrum of the CO-reduced form. Concentrations of mutant proteins were determined using molar absorption coefficients of 1.09×10^5 M^{-1} cm^{-1} at 393 nm for the high-spin oxidized form and 9.3×10^4 M^{-1} cm^{-1} at 447 nm for the [CO-reduced] – [reduced] difference spectra (Shimizu et al., 1991a).

NADPH-cytochrome P450 reductase was purified from phenobarbital-induced rat liver microsomes with DEAE-Toyopearl (Tosoh Co., Tokyo, Japan) and ADP–Sepharose (Pharmacia LKB Biotech, Uppsala, Sweden) as previously described (Iyanagi et al., 1981).

Rates of NADPH oxidation, O_2 consumption, H_2O_2 formation, and product formation (turnover number) were obtained in the reconstituted system consisting of 0.5 mM 7-ethoxycoumarin or 1 M methanol, 0.3 μM P450 1A2, 0.9 μM NADPH-P450 reductase, 1 mM NADPH, 48 μM DLPC, 0.1 M potassium phosphate (pH 7.4), 4% glycerol (v/v), 0.2 mM EDTA, and 0.2 mM DTT at 25 °C. The presence of 4% glycerol (v/v) did not affect the various rates. By increasing the reductase concentration up to 3 μM, turnover numbers of the wild type and mutants were not essentially changed. Rates of NADPH oxidation were monitored by the absorption of NADPH at 365 nm ($\epsilon = 3.5 \times 10^3$ M^{-1} cm^{-1}) for 7-ethoxycoumarin hydroxylation and at 340 nm ($\epsilon = 6.2 \times 10^3$ M^{-1} cm^{-1}) for methanol hydroxylation (Hiroya et al., 1992) at 25 °C. Rates of O_2 consumption were determined with a Clark-type oxygen electrode Oxygraph-9, UD-1+UD-901(Central Kagaku Corp., Tokyo, Japan) at 25 °C (Hiroya et al., 1992; Ishigooka et al., 1992). Rates of H_2O_2 formation were obtained by using a H_2O_2 detection kit, DA-64 (Wako Pure Chemical Industry, Osaka, Japan) in the presence of peroxidase (Hiroya et al., 1992). Turnover numbers toward 7-ethoxycoumarin in the reconstituted system were obtained by monitoring the fluorescence emission band at 458 nm (excited at 368 nm) of the product after double extractions with chloroform and subsequent triple extractions with borate buffer (pH 9.5) (Guengerich, 1978). Turnover numbers toward methanol in the reconstituted system were obtained from formaldehyde formation with Nash reagent (Hiroya et al., 1992). Turnover numbers in the *tert*-butyl hydroperoxide system were obtained for 0.5-mL solutions consisting of 0.3 μM P450 1A2, 0.5 mM 7-ethoxycoumarin or 1 M methanol, 48 μM DLPC, 10 mM *tert*-butyl hydroperoxide, 0.2 mM EDTA, 4% glycerol (v/v), and 0.1 M potassium phosphate (pH 7.4) at 25 °C. Reactions were terminated by adding 2 N HCl after incubation for 20 or 15 min. Other procedures were the same as those in the reconstituted system. Degradation of the heme of P450 1A2 caused by *tert*-butyl hydroperoxide was very slow (4.0×10^{-6} sec^{-1} mM^{-1}) at 25 °C (our unpublished results).

Rate constants of the P450 reduction were obtained from the fast phase by monitoring the absorption at 447 nm of the CO-reduced complexes (Shimizu et al., 1991b; Strobel et al., 1970). Those values were obtained for the oxygen-free 40-μL solution consisting of 0.15 μM P450 1A2, 0.45 μM reductase, 48 μM DLPC, 0.5 mM NADPH, 0.2 mM EDTA, 4% glycerol (v/v), 30 mM glucose, 50 units/mL glucose oxidase, 1000 units/mL catalase, approximately 0.8 mM CO, and 0.1 M potassium phosphate (pH 7.4) at 25 °C. Increasing the reductase concentration up to 1.5 μM did not essentially change the rate constants of the wild type and the mutants.

Apparent spectral dissociation constants (K_d) for dissociation of the reductase from P450 1A2 were obtained by monitoring the spectral change of the Soret band of P450 1A2. Partial low-spin P450 1A2 with a Soret absorption peak at 416 nm in the presence of 20 mM acetanilide was changed to a high-spin complex with an absorption peak at 393 nm by adding the reductase (Shimizu et al., 1991a,b). The spectral change from low-spin to high-spin complexes was fitted to the Michaelis–Menten equation under the assumption that a 1:1 P450–reductase complex is formed (Shimizu et al., 1991b).

Phospholipids, horseradish peroxidase (Type VI-A), catalase from bovine liver, and metmyoglobin from horse heart were purchased from Sigma Chemical Co. (St. Louis, MO). Glucose oxidase from *Aspergillus niger* was purchased from Wako Pure Chemical Industry (Osaka, Japan). 7-Ethoxycoumarin was obtained from Aldrich Chemical Co. (Milwaukee, WI). Hemin was obtained from Tokyo Kasei Co. (Tokyo, Japan). *tert*-Butyl hydroperoxide was obtained from Merck-Schuchardt (Munich, FRG). Other chemicals used were of the highest guaranteed grade and were used without further purification.

Optical absorption spectra were obtained with Shimadzu UV-365 and UV-2200 UV spectrometers. Rate constants of the reduction of the heme of the mutants were obtained on a Photal RA401 stopped-flow spectrophotometer (Otsuka Electronics Co., Osaka, Japan) equipped with micromixing cells and directly connected to a NEC PC-9801DX personal computer (Shimizu et al., 1991b).

RESULTS

Catalytic Activities toward 7-Ethoxycoumarin. The turnover number (0.02 min^{-1}) of Lys99Glu, Lys453Glu, and

Table I: Stoichiometry of *O*-Deethylation Reactions toward 7-Ethoxycoumarin by P450 1A2 Mutants in the Presence of NADPH–Cytochrome P450 Reductase[a]

P450 1A2	rate (nmol/nmol P450/min) NADPH (A)	O_2 (B)	H_2O_2	product (C)	C/A (%)	C/B (%)
wild type	29	25	13	0.35	1.2	1.4
control[b]	2.0	1.5	1.7	<0.01		
Lys99Glu	7.8	6.7	5.7	0.02	0.3	0.3
Arg137Leu	18	22	11	0.18	1.0	0.8
Lys401Glu	36	17	9.1	0.35	1.0	2.1
Lys453Glu	8.8	7.9	2.6	0.02	0.2	0.3
Arg455Glu	11	11	6.8	0.02	0.2	0.2
hemin[c]				<0.01		
metMb[c]				<0.01		
HRP[c]				<0.01		

[a] Experiments were repeated at least three times. Experimental errors were less than 20%. [b] Control values were obtained under the same conditions in the absence of P450 1A2. Rates are expressed as nmol/nmol reductase/min. [c] Turnover numbers of hemin, metmyoglobin (metMb), and horseradish peroxidase (HRP) were obtained in the same way as for P450 1A2 enzymes and are expressed as nmol/nmol heme/min. Heme concentrations used were 0.3 μM.

Table II: Stoichiometry of Hydroxylation Reactions toward Methanol by P450 1A2 Mutants in the Presence of NADPH–Cytochrome P450 Reductase[a]

P450 1A2	rate (nmol/nmol P450/min) NADPH (A)	O_2 (B)	H_2O_2	product (C)	C/A (%)	C/B (%)
wild type	63	34	15	4.7	7.5	14
control[b]	0.97	0.64	1.6	0.10	10	16
Lys99Glu	7.9	7.2	5.4	0.60	7.6	8.3
Arg137Leu	46	28	19	2.1	4.6	7.5
Lys401Glu	74	52	30	4.8	6.5	9.2
Lys453Glu	11	7.9	1.3	0.82	7.5	10
Arg455Glu	11	11	6.1	0.66	6.0	6.0
hemin[c]				0.45 (0.15)[d]		
metMb[c]				0.26 (0.09)[d]		
HRP[c]				0.35 (0.12)[d]		

[a] Experiments were repeated at least three times. Experimental errors were less than 20%. [b] Control values were obtained under the same conditions in the absence of P450 1A2. Rates are expressed as nmol/nmol reductase/min. [c] Turnover numbers of hemin, metmyoglobin (metMb), and horseradish peroxidase (HRP) were obtained under the same conditions as for P450 1A2 enzymes and are expressed as nmol/nmol heme/min. Heme concentrations used were 0.3 μM. [d] Turnover numbers are expressed as nmol/nmol reductase/min.

Table III. Rate Constants (s^{-1}) of Electron Transfer and Spectral Dissociation Constants (K_d) for P450 1A2 Mutants from NADPH–Cytochrome P450 Reductase

P450 1A2	rate constant[a] (s^{-1})	K_d[b] (nM)	ref
wild type	5.2	47	Shimizu et al. (1991b)
Lys99Glu	0.08	210	Shimizu et al. (1991b)
Arg137Leu	2.4	40	this work
Lys401Glu	3.1	70	this work
Lys453Glu	0.05	>200	Shimizu et al. (1991b)
Arg455Glu	0.05	<28	Shimizu et al. (1991b)

[a] Experiments were repeated at least six times. Experimental errors were less than 30%. [b] Spectral dissociation constants (K_d) of the reductase were obtained in the presence of 20 mM acetanilide as previously described (Shimizu et al., 1991b). Experiments were repeated twice. Experimental errors were less than 20%.

Arg455Glu mutants in the reconstituted system in the presence of excess reductase (from more than 3:1 up to 10:1 molar ratios of reductase to P450) was much lower than that (0.35 min^{-1}) of the wild type (Table I). The turnover number (0.35 min^{-1}) of the Lys401Glu mutant was comparable to that of the wild type, while that (0.18 min^{-1}) of the Arg137Leu mutant was one-half that of the wild type. Other heme complexes such as hemin, metmyoglobin, and horseradish peroxidase did not show any activity toward 7-ethoxycoumarin in the presence of the excess reductase and NADPH (Table I).

The catalytic parameters, rate of oxidation of NADPH, consumption of O_2, and production of H_2O_2, of the Lys99Glu, Lys453Glu, and Arg455Glu mutants were lower than those of the wild type (Table I), but the differences in these parameters between the mutants and the wild type were not so remarkable as those observed for the turnover numbers of the corresponding mutants. The catalytic parameters of the Arg137Leu and Lys401Glu mutants were comparable to those of the wild type (Table I). Catalytic efficiencies, percentages of rates of product formation per NADPH oxidized, and rates of product formation per consumed O_2 for the Lys99Glu, Lys453Glu, and Arg455Glu mutants were much lower than those of the wild type, while those of Arg137Leu and Lys401Glu mutants were comparable to those of the wild type (Table I).

Catalytic Activities toward Methanol. Turnover numbers (0.60–0.82 min^{-1}) of the Lys99Glu, Lys453Glu, and Arg455Glu mutants toward methanol were lower than that (4.65 min^{-1}) of the wild type (Table II), but these differences between the mutants and the wild type were not so remarkable as those observed for the hydroxylation rates toward 7-ethoxycoumarin (Table I). The turnover number (4.82 min^{-1}) of the Lys401Glu mutant was comparable to that of the wild type, while that of the Arg137Leu mutant was 46% that of the wild type (Table II). Other heme complexes had catalytic activities toward methanol to a certain extent, which is in contrast with those observed for 7-ethoxycoumarin (Table I).

Catalytic parameters of the reaction for the Lys99Glu, Lys453Glu, and Arg455Glu mutants were smaller than those of the wild type, which correspond to the turnover numbers of the methanol hydroxylation (Table II). Catalytic efficiencies, percentages of rates of product formation per NADPH oxidized, and rates of product formation per consumed O_2 for these three ionic mutants were not remarkably different from those of the wild type, which is in contrast with those observed for 7-ethoxycoumarin hydroxylation (Table I). Catalytic parameters of the Arg137Leu and Lys401Glu mutants correspond to the turnover numbers of the methanol hydroxylation (Table II).

Effects of the Substrates on the Rate Constant of Electron Transfer. Rate constants (0.05–0.08 s^{-1}) of the Lys99Glu, Lys453Glu, and Arg455Glu mutants in the presence of excess reductase (from more than 3:1 up to 10:1 molar ratios of reductase to P450 1A2) were much lower than that (5.22 s^{-1}) of the wild type, while those (2.41–3.07 s^{-1}) of the Arg137Leu and Lys401Glu mutants were comparable to that of the wild type (Table III). From the above-mentioned findings about catalytic activities, however, it was thought that the electron-transfer mechanism and/or the interaction between P450 and the reductase may be different in the presence of 7-ethoxycoumarin or methanol. Thus, we studied whether or not the rate constant of electron transfer from the reductase to the heme of the wild type is influenced by the substrate. Addition of 0.5 mM 7-ethoxycoumarin to the reconstituted solution in the absence of the substrate did not change the rate constants of electron transfer. In contrast, addition of methanol from 0.3 M up to 1.0 M to the reconstituted solution notably decreased the rate constant by 30% (Figure 1B), while the turnover number of the wild type increased by raising the

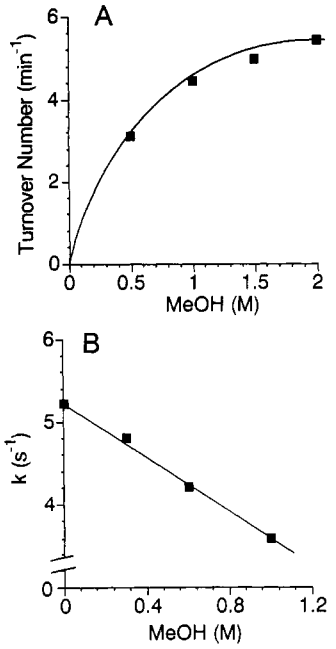

FIGURE 1: Effect of methanol concentration on the turnover numbers of methanol hydroxylation (A) and the electron-transfer rate constants (B) of wild-type P450 1A2. The experimental conditions are described in Experimental Procedures.

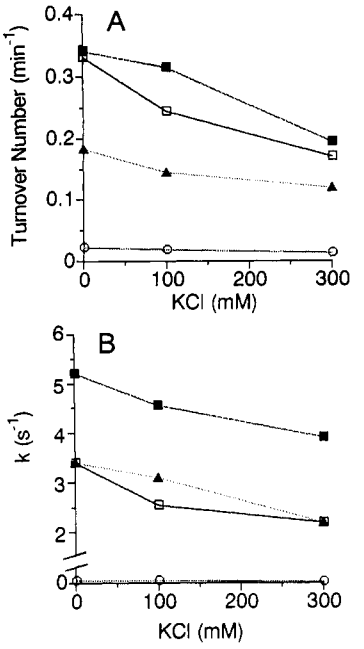

FIGURE 2: Turnover numbers of 7-ethoxycoumarin hydroxylation (A) and rate constants of electron transfer (B) of wild-type and mutant enzymes in the presence of various amounts of KCl. The experimental conditions are described in Experimental Procedures except that 50 mM potassium phosphate buffer (pH 7.4) was used in this study. Rate constants expressed as s^{-1} are in some cases a little different from those obtained in 0.1 M potassium phosphate buffer (pH 7.4), described in Table IV. Symbols: (■) wild type; (□) Lys401Glu mutant; (▲) Arg137Leu mutant; (○) Lys453Glu mutant. Lys99Glu and Arg455Glu mutants showed essentially the same values as those of the Lys453Glu mutant.

methanol concentration (Figure 1A). Thus, the whole catalytic activity of P450 1A2 toward methanol does not seem to be necessarily ascribable to direct electron transfer from the reductase.

Effect of KCl on the Turnover Number and the Rate Constant of Electron Transfer. We obtained turnover numbers of the wild type and the mutants in the presence of excess reductase (3:1 molar ratio of reductase to P450) and various concentrations of KCl (Figure 2A). The turnover number of the wild type toward 7-ethoxycoumarin was decreased by approximately 40% by increasing the concentration of KCl from 0 to 300 mM. The same tendency was observed for the Arg137Leu and Lys401Glu mutants as well. The low turnover numbers for the Lys99Glu, Lys453Glu, and Arg455Glu mutants did not increase by raising KCl concentration. The turnover number of the wild type toward methanol was decreased by raising the KCl concentration in the same way as observed for 7-ethoxycoumarin.

The electron-transfer rate constants of the wild type and the Arg137Leu and Lys401Glu mutants were decreased by 25% by increasing the KCl concentration (Figure 2B), which is in accordance with the changes observed for the turnover numbers (Figure 2A). The low electron-transfer rate constants of the Lys99Glu, Lys453Glu, and Arg455Glu mutants did not change by increasing the concentration of KCl (Figure 2B). The lowered rate constant caused by 1 M methanol in the reconstituted solution composed of the wild type and the reductase was not changed by raising the KCl concentration.

Spectral Dissociation Constants. Apparent spectral dissociation constants (K_d) of the reductase dissociation from the wild-type and mutant enzymes in the presence of 20 mM acetanilide were obtained (Shimizu et al., 1991b) (Table III). The K_d values for the newly constructed Arg137Leu and Lys401Glu mutants were not different from that of the wild type.

Catalytic Activities Caused by tert-Butyl Hydroperoxide. The turnover number (0.79 min^{-1}) toward 7-ethoxycoumarin for the wild type caused by *tert*-butyl hydroperoxide in the absence of the reductase and NADPH (Table IV) was more than 2-fold that obtained in the reconstituted system consisting of NADPH and the reductase (Table I). On the other hand, the turnover number (0.70 min^{-1}) toward methanol of the wild type in the hydroperoxide system was only 15% that obtained in the reconstituted system (Table II). Turnover numbers (0.32–0.65 min^{-1}) for most ionic mutants toward 7-ethoxycoumarin in the hydroperoxide system were only a little lower than that (0.79 min^{-1}) of the wild type, which is in contrast with those observed in the reconstituted system. Turnover numbers (0.49–0.65 min^{-1}) toward methanol in the hydroperoxide system for some mutants were also only a little lower than that (0.70 min^{-1}) of the wild type. These differences (Table IV) in turnover numbers toward both substrates between the wild type and the ionic mutants Lys99Glu, Lys453Glu, and Arg455Glu in the hydroperoxide system were

Table IV: Turnover Numbers (nmol/nmol P450/min) of P450 1A2 Mutants toward 7-Ethoxycoumarin and Methanol in the Presence of *tert*-Butyl Hydroperoxide[a]

P450 1A2	7-ethoxycoumarin	methanol
wild type	0.79	0.70
control[b]	<0.01	0.03
Lys99Glu	0.38	0.57
Arg137Leu	0.32	0.65
Lys401Glu	0.39	0.99
Lys453Glu	0.83	0.78
Arg455Glu	0.65	0.49
hemin[c]	<0.01	0.14
metMb[c]	<0.01	0.21
HRP[c]	<0.01	0.15

[a] Experiments were repeated at least three times. Experimental errors were less than 20%. [b] Control values were obtained under the same conditions in the absence of P450 1A2 enzyme. [c] Turnover numbers of hemin, metmyoglobin (metMb), and horseradish peroxidase (HRP) were obtained in the same way as P450 1A2 enzymes and are expressed as nmol/nmol heme/min. Heme concentrations used were 1 μM.

Table V: Effect of Phospholipids on Activity of Wild-Type P450 1A2 toward 7-Ethoxycoumarin[a]

phospholipids	turnover (%)	phospholipids	turnover (%)
(1) DLPC	100	(1) + (2)	84
(2) L-α-phosphatidyl-DL-glycerol	94	(1) + (3)	87
(3) L-α-phosphatidylcholine	91	(1) + (4)	104
(4) dipalmitoyl-*N*-dansyl-L-α-phosphatidylethanolamine	80	(1) + (5)	89
		(1) + (6)	100
(5) L-α-phosphatidylethanolamine	94	(3) + (4)	79
(6) dioleoyl-L-α-phosphatidylcholine	103	(3) + (5)	77
(7) L-α-phosphatidyl-L-serine	79	(3) + (6)	99
(8) dimyristoyl-L-α-phosphatidic acid	78	(4) + (5)	74
		(4) + (6)	93
(9) dimyristoyl-L-α-phosphatidylethanolamine	78	(5) + (6)	95
(10) dipalmitoyl-DL-α-phosphatidylcholine	75		

[a] Turnover numbers were obtained in the same way as in Table I. The concentration of phospholipids was 48 μM.

apparently much less remarkable than those observed in the reconstituted system (Table I). Hemin, metmyoglobin, and horseradish peroxidase did not catalyze the hydroxylation toward 7-ethoxycoumarin in the presence of *tert*-butyl hydroperoxide, while these compounds did catalyze methanol oxidation to a certain extent (Table IV).

Effect of Phospholipids on Catalytic Activities. It is conceivable that the interaction between P450 1A2 and the reductase is influenced by the components of phospholipids in the reconstituted system (French et al., 1980; Müller-Enoch et al., 1984; Imaoka et al., 1992). We checked the effects of phospholipids on the turnover numbers toward 7-ethoxycoumarin of the wild-type P450 1A2 (Table V). Turnover numbers were not so different (less than 25%) among phospholipids studied. We obtained the highest turnover number when DLPC alone was used in the reconstituted system.

DISCUSSION

For the Lys99Glu, Lys453Glu, and Arg455Glu mutants, the catalytic activities toward 7-ethoxycoumarin and the rate constants of electron transfer from the reductase were remarkably smaller than those of the wild type. It is interesting to note that catalytic efficiencies for the reactions of those ionic mutants were also much lower than that of the wild type, in correspondence with the turnover numbers. Similar results were observed for methanol hydroxylation by those mutants. Namely, the electron transfer and the O_2 consumption of the ionic mutants are well associated with the turnover number in the same way as observed for the wild type. This finding indicates that catalytic reactions of P450 1A2 are coupled to a certain extent even in the microsomal system, although overall catalytic reactions are not strictly coupled with electron transfer and O_2 consumption. Thus the uncoupling of the microsomal P450 reactions is neither due to a weak interaction with the reductase nor due to leakage of the electrons from the system, but may be due to inefficient monooxidation reactions at the heme active site.

Catalytic efficiencies and effects of mutations of the ionic amino acids of P450 1A2 for methanol hydroxylation were apparently higher than those of 7-ethoxycoumarin hydroxylation (Tables I and II). The electron-transfer mechanism in the hydroxylation reaction and/or the interaction between P450 1A2 and the reductase may be different between the 7-ethoxycoumarin and methanol systems. In fact, the rate constant of electron transfer from the reductase to P450 1A2 was remarkably reduced by methanol (Figure 1B), while it was not essentially influenced by 7-ethoxycoumarin. Methanol may influence appropriate geometries or fluctuations of both enzymes which are optimum for the electron transfer. Perhaps excess methanol impedes the interaction between P450 1A2 and the reductase. How, then, is catalytic function toward methanol caused by the slow electron transfer? In order to identify compound(s) that contribute to methanol hydroxylation, we added hydroxyl radical scavengers, mannitol and dimethyl sulfoxide, and superoxide dismutase (Ingelman-Sundberg & Johansson, 1981; Cederbaum, 1983) to the reconstituted solutions containing 7-ethoxycoumarin or methanol. Turnover numbers of the wild-type P450 1A2 toward both substrates were not changed by those effectors. However, the turnover number of the wild-type P450 1A2 toward methanol in the reconstituted system was increased 7-fold by adding catalase, while that toward 7-ethoxycoumarin was not changed by adding catalase (our unpublished results). Catalase itself catalyzes the methanol hydroxylation by using H_2O_2 (Hildebrandt & Roots, 1975). The reductase itself produces H_2O_2 from O_2 to a certain extent (Tables I and II). Thus, it seems likely that H_2O_2 produced by the reductase itself may contribute to methanol hydroxylation in the reconstituted system of P450 1A2 (Ortiz de Montellano, 1986). This H_2O_2-supported reaction toward methanol will be enhanced by non-P450 iron complexes (Table II) (Ingelman-Sundberg & Johansson, 1984). Note that degradation of the heme of P450 1A2 caused by H_2O_2 (Schaefer et al., 1985) during the hydroxylation reactions in the reconstituted system at 25 °C for 20 min was about 25% in terms of absorption spectra of the CO-reduced form. The contribution by the H_2O_2-supported methanol hydroxylation relatively increases concomitant with increase of the methanol concentration as the electron-transfer rate from the reductase decreases (Figure 1). Thus, it seems likely from the present findings that the electron-transfer mechanism from the reductase to P450 1A2 and/or the interaction between the two proteins, and the mechanism of the hydroxylation reactions, may be different between the 7-ethoxycoumarin and methanol systems.

It was claimed from chemical modification studies (Bernhardt et al., 1984, 1988; Shen & Strobel, 1992) that the well-conserved Lys384 of P450 2B4, or Lys407 of P450 1A1, which corresponds to Lys401 of P450 1A2 (Nelson & Strobel, 1988; Poulos et al., 1985, 1987), is important for the interaction with the reductase. However, it is suggested in the present study that the well-conserved Lys401 of P450 1A2 is not directly involved in the interaction with the reductase. This Lys401, which corresponds to Lys466 (specially numbered for all P450s) in Nelson and Strobel (1988), seems to be located at the opposite end of ionic patches of the protein molecule, from the crystal structure of $P450_{cam}$ (Poulos et al., 1985, 1987; Nelson & Strobel, 1988).

Turnover numbers toward 7-ethoxycoumarin and methanol (Tables I and II) and the electron-transfer rate constant (Table III) for the mutant Arg137Leu in the reconstituted system were nearly one-half those of the wild type (Table I). Thus, it seems that the conserved Arg137 itself may play a certain role in the interaction with the reductase. The Arg cluster Arg135-Arg136-Arg137 *per se* of P450 1A2 may perhaps participate in the interaction with the reductase, since catalytic activity of the triple mutant toward 7-ethoxycoumarin was less than 10% that of the wild type in the microsomal system (Shimizu et al., 1991b).

It is suggested from the present KCl-dependent studies that ionic or electrostatic character is important, if modestly, in the interaction of P450 1A2 with the reductase, since catalytic activities toward 7-ethoxycoumarin and rate constants of the

electron transfer decreased when the KCl concentration was raised (Figure 2). A similar decrease in the turnover number toward methanol hydroxylation was observed when the KCl concentrations were raised. If hydrophobic character is dominant in the protein–protein interaction, addition of KCl to the mutant–reductase solutions must increase the interaction between the two proteins. Addition of excess KCl to the mutant–reductase solutions, however, did not increase the catalytic activities or the electron-transfer rate constants for any mutant enzymes studied here. Thus it is suggested here that the interaction of P450 1A2 with the reductase is rather ionic, if modestly, composed of several ionic interactions between the two proteins as previously implied (Bernhardt et al., 1984, 1988; Nadler & Strobel, 1988, 1991). This result is different from that observed for $P450_{cam}$ in that the apparent rate constant of the reduction of $P450_{cam}$ by putidaredoxin was not changed until the KCl concentration reached 0.5 M (Brewer & Peterson, 1988), suggesting that the $P450_{cam}$–putidaredoxin interaction may be less ionic (Brewer & Peterson, 1988; Stayton et al., 1989). In addition, when the concentration of sodium phosphate was increased in the P450 2B4–reductase system, the electron-transfer rate and the catalytic activities were remarkably enhanced (Voznesensky & Schenkman, 1992). Thus, the ionic character of the P450 1A2–reductase system may be different from those for the $P450_{cam}$ and P450 2B4 systems. Nevertheless, it should be noted that certain contribution of hydrophobicity to the interaction between P450 1A2 and reductase cannot be totally ruled out in the present study, since methanol, a rather hydrophobic compound, seems to impede the interaction between the two enzymes as mentioned above.

Apparent dissociation constants (K_d) for dissociation of the Lys99Glu and Lys453Glu mutants from the reductase were higher than that of the wild type (Table III). Lys99 and Lys453 thus seem to be involved in the interaction with the reductase or in anchoring the reductase rather than directly participating in the electron transfer between the two proteins. The turnover numbers and the electron-transfer rate constant of the Arg455Glu mutant were remarkably lower than those of the wild type even though this mutant has higher affinity for the reductase than the wild type (Table III). Perhaps Arg455 may be important for keeping a proper orientation and geometry for efficient electron transfer to occur. Arg137 and Lys401 may not be important in the interaction with the reductase.

In chemical modification studies of the interaction between P450 and reductase, hydroperoxide compounds were used to prove that the heme active site and the substrate-binding site are not largely destroyed by chemical modifications (Shen & Strobel, 1992). In fact, the kinetic parameters k_{cat} and K_m of P450 2D1 in the peroxide-supported system are remarkably varied by mutations at the putative substrate-recognition site of the enzyme quite in correspondence with those in the reductase-supported system (Matsunaga et al., 1990). The kinetic parameters of the 7-ethoxycoumarin *O*-deethylation reaction in the *tert*-butyl hydroperoxide-supported system are also remarkably changed by mutations at the putative distal site of P450 1A2 (our unpublished results). Thus, to determine whether or not the heme active site and a substrate-binding site(s) of P450 1A2 are changed by the mutations of ionic amino acids, we obtained turnover numbers of the mutants caused by *tert*-butyl hydroperoxide in the absence of the reductase and NADPH (Nordblom et al., 1976). The catalytic activities of those mutants caused by *tert*-butyl hydroperoxide in the absence of the reductase and NADPH were essentially the same as that of the wild type (Table IV). This finding may suggest that the heme active site and a substrate-binding site(s) of those mutants are not largely destroyed by those mutations. Since hemin, metmyoglobin, and horseradish peroxidase did not catalyze the hydroxylation of 7-ethoxycoumarin in the presence of *tert*-butyl hydroperoxide, the heme environment of P450 1A2 is important for the catalytic function toward 7-ethoxycoumarin.

At least in the reconstituted system, constituents of phospholipids do not seem important in the interaction between P450 1A2 and reductase and in electron transfer from the reductase to the heme of P450 1A2 (Table V).

In conclusion, the present study suggests the following: (1) The coupling efficiency of the hydroxylation reaction toward 7-ethoxycoumarin of P450 1A2 in the reconstituted system was remarkably reduced when Lys99, Lys453, and Arg455 were mutated to Glu. However, this reduction is moderate when methanol is used as a substrate. Thus, the electron-transfer mechanism and/or the interaction between P450 1A2 and the reductase in the presence of 7-ethoxycoumarin may be somehow different from those in the presence of methanol. This was partially verified by the fact that the electron-transfer rate constant is notably different between the 7-ethoxycoumarin and methanol systems. Nonenzymatic contribution by H_2O_2 as a byproduct to the methanol hydroxylation is implied. (2) The well-conserved Lys401 of P450 1A2 is not important for the interaction with the reductase. (3) Ionic character is important, if modestly, in the interaction between P450 1A2 and the reductase. (4) Mutations of these ionic amino acids do not seem to essentially change the structure of the heme active site and a substrate-binding site(s) in terms of catalytic activities caused by *tert*-butyl hydroperoxide.

The fraction of electrons devoted to product formation in these reactions is extremely low. Substrates such as acetanilide and certain arylamines with high rates of product formation might be more appropriate for use in these studies.

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