Roles of Residues 129 and 209 in the Alteration by Cytochrome *b*5 of Hydroxylase Activities in Mouse 2A P450S

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ABSTRACT: Cytochrome *b*5 stimulates the coumarin 7-hydroxylation activity of P450coh. A mutation of Arg-129 in P450coh, however, abolishes the stimulation. Moreover, this mutant P450coh binds loosely to cytochrome *b*5-conjugated Sepharose 4B, whereas wild-type P450coh binds tightly. Consistent with this, the mutation increases the K_a value for *b*5 binding approximately 6-fold. The identity of residue 209 also alters the stimulation of the activity of P450coh depending on the type of the substrates used and products formed. Coumarin 7-hydroxylation activity is greatly stimulated by cytochrome *b*5 only when Phe is at position 209, while cytochrome *b*5 stimulates testosterone hydroxylation activity of P450coh in which Phe, Asn, Ser or Lys substitutes residue 209. P450coh changes its rate of hydrogen peroxide formation depending on the identity of residue 209 and substrate used. Cytochrome *b*5 decreases the hydrogen peroxide formation of some P450coh whose activities are stimulated by the cytochrome; however, the decrease does not always result in stimulating the activity. The results indicate, therefore, that residues 129 and 209 play different roles in stimulating P450coh activity by cytochrome *b*5; Arg-129 is a key residue in the cytochrome *b*5-binding domain and is essential for the stimulation. Residue 209, however, alters the efficiency of electron transport for substrate oxidation as a residue which resides near the sixth ligand of heme and in the substrate-binding site.

P450 is a terminal monoxygenase of the membrane-bound electron transport system consisting of NADPH–P450 reductase, NADH–cytochrome *b*5 reductase, and cytochrome *b*5 (*b*5). Two electrons are necessary to reduce P450 and can be donated in two ways; both can be transferred from NADPH–P450 reductase, or the first electron can come from the reductase and the second one from *b*5 (Ruckpaul & Rein, 1989; Tamburini et al., 1985). This *b*5 donation, therefore, can alter and often results in stimulating the activities of various P450s (Aoyama et al., 1990; Ingelman-Sundber et al., 1980; Jansson et al., 1985; Usanov et al., 1990). The stimulation, however, differs greatly depending on the identity of the P450s, substrates, and products. Locating a *b*5–P450 interaction site, therefore, is an important step in understanding the complex mechanisms of *b*5-dependent stimulation of P450 activity. Recently, Jansson et al. (1987) suggested that a *b*5-binding site in rat P4502B1 is nested within the recognition motif of cAMP-dependent protein kinase (PKA), since *b*5 competitively inhibits the phosphorylation of the P450 (Jansson et al., 1987). A positively-charged amino acid in the motif Arg-Arg-X-Ser is an ideal candidate for the binding site of negatively-charged *b*5 (Tamburini et al., 1985). Moreover, Stayton and Sligar (1990) has suggested the interactions of Lys-344, Arg-72, Arg-112, and Arg-346 in P450cam with *b*5.

Mouse P450coh (2A5) and $P450_{15\alpha}$ (2A4) catalyze coumarin 7-hydroxylation and testosterone 15α-hydroxylation activities, respectively, although they differ only by 11 residues within their 494 amino acids (Lindberg & Negishi, 1989; Negishi et al., 1989). Interestingly, one of the 11 different residues resides in the PKA recognition motif; P450coh contains the site (^{128}Arg-Arg-Phe-^{131}Ser), whereas Arg-129 is mutated to serine in $P450_{15\alpha}$. In addition to this sequence difference, *b*5 increases the coumarin 7-hydroxylation activity of P450coh (Usanov, 1990), while the testosterone 15α-hydroxylation activity of $P450_{15\alpha}$ is decreased by *b*5 (Harada & Negishi, 1984). It is not known, however, whether Arg-129 plays a role in regulating the effect of *b*5 on the activity of the P450s.

Residue 209 is most critical in determining the substrate specificity of P450coh and $P450_{15\alpha}$, because its identity alters the specificity of P450coh from coumarin 7- to steroid 15α-hydroxylation activity (Lindberg & Negishi, 1989). Our site-directed mutagenesis studies indicate that residue 209 resides close to a substrate-binding site and to the sixth ligand of heme in the P450s (Iwasaki et al., 1991; Juvonen et al., 1991). The P450coh and $P450_{15\alpha}$ system, therefore, provides an excellent model to study not only the *b*5–P450 interaction but also the effect of the structural alteration of the substrate heme pocket on the *b*5-dependent stimulation of P450 activity.

This paper examines the roles of Arg-129 and residue 209 in the stimulation of P450coh activity by *b*5. For this purpose, we genetically engineered mutants of P450coh and studied their biochemical and enzymological properties. As a consequence, we propose that Arg-129 is the key residue in the *b*5-binding domain in P450coh. Moreover, the identity of residue 209, at least in part, regulates the substrate and product specificity of *b*5-stimulated activity of the P450.

MATERIALS AND METHODS

Chemicals. We obtained coumarin and 7-hydroxycoumarin from Aldrich Chemical Co., Inc. (Milwaukee, WI), CNBr-activated Sepharose 4B from Pharmacia LKB Biotechnology (Uppsala, Sweden), 7-ethoxycoumarin, dilauroylphosphatidylcholine, and NADPH from Sigma Chemical Co. (St. Louis, MO, [4-^{14}C]testosterone from Amersham Corp.; the enzymes and reagents for molecular biology from Stratagene (La Jolla, CA), New England Biolabs (Beverly, MA) or Promega (Madison, WI), and polymerase chain reaction kits from Perkin Elmer Cetus (Norwark, CT).

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Expression of P450 in Yeast Cells. We expressed in *Saccharomyces cerevisiae* AH22 cells the wild-type P450coh, $P450_{7\alpha}$, and $P450_{15\alpha}$ as previously described (Iwasaki et al., 1991; Juvonen et al., 1991; Oeda et al., 1985; Iwasaki et al., 1992). Also, the construction of mutants P450coh was reported in our previous papers (Lindberg & Negishi, 1989; Iwasaki et al., 1991; Juvonen et al., 1991), except mutants R129S and H320Y, in which Arg-129 or His-320 was replaced by serine or tyrosine. For these, we amplified the already mutated cDNAs (Lindberg & Negishi, 1989) using 5′-GCAAGCTTAAAAAAATGCTGACCTCAGGA and 5′-CGAAGCTTTGTGTCCTCTGTTTCTTCT as the 5′- and 3′-primers, respectively. Newly added *Hin*dIII sites are underlined. The amplified cDNAs were digested by *Hin*dIII, ligated to pAAH5 vectors, and then transfected into *S. cerevisiae* AH22 as described by Oeda et al. (1985).

Purification of P450 and Other Enzymes. We prepared microsomes from the recombinant yeast cells and purified the P450s using the methods previously described (Oeda et al., 1985; Iwasaki et al., 1991). The purified P450s were stored in 100 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.4% sodium cholate, 0.2% Emulgen 913, 1 mM EDTA, and 1 mM DTT. The method of Yasukochi and Masters (1976) and its modifications (Tamburini & Gibson, 1983; Usanov et al., 1990) were used to purify NADPH–P450 reductase and cytochrome *b*5 from phenobarbital-treated Fisher male rats and female CD-1 mice. Purified *b*5 was dialyzed against 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 0.2% cholate, and frozen at −70 °C.

Affinity Chromatography. We conjugated rat *b*5 with Sepharose 4B using the method of Kawata et al. (1986). Briefly, CNBr-activated Sepharose 4B (0.3 g) was first washed with 1 mM HCl, then with 100 mM carbonate buffer, pH 8.3, containing 500 mM NaCl, and finally incubated with rat *b*5 (24 μmol) in 2 mL of the carbonate buffer overnight at 4 °C. The *b*5-conjugated Sepharose 4B was washed with 10 mM Tris-HCl buffer, pH 7.4, containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.2% Emulgen 913, and 1 M NaCl and then equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.2% Emulgen 913. Purified P450coh (200–300 pmol) was dialyzed against the equilibrium buffer and applied on the *b*5–Sepharose 4B column and eluted by the stepwise increase of NaCl concentration in the buffer. Coumarin 7-hydroxylase activity in each fraction collected was measured to obtain the elution profile of P450coh from the column.

b5-Induced Difference Spectra. The purified wild-type P450coh and mutant R129S were dialyzed against 10 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.2% Emulgen 913. The P450 solution was divided into a tandem cuvette and incubated with various amounts of rat *b*5 to produce difference spectra. The amounts of *b*5 were 20, 40, 81, 160, 320, 620, and 1200 μM.

Analytical Methods. P450 content, coumarin 7-hydroxylation, 7-ethoxycoumarin O-deethylation, and testosterone hydroxylation activities were determined by the methods of Omura and Sato (1964), Aitio (1978), Juvonen et al. (1988), Juvonen et al. (1991) and Harada and Negishi (1984), respectively. Unless specified, standard reconstitution assays consisted of P450 (10 pmol), NADPH–P450 reductase (30 pmol), dilauroylphosphatidylcholine (6 nmol), $MgCl_5$ (5 mM), NADPH (0.5 mM), and 100 μM coumarin in 0.5 mL of 50 mM potassium phosphate buffer, pH 7.4, or 100 μM testosterone in 0.5 mL of 50 mM Tris-HCl buffer, pH 7.5. Hydrogen peroxide formation was measured by the ferrithiocyanate method (Thurman et al., 1972). We repeated this in the presence of 100 μM desferrioxamine to decrease the background by transition metals (Morehouse et al., 1984).

Table I: Effects of *b*5 on the Enzymatic Kinetics of P450coh, $P450_{7\alpha}$, and $P450_{15\alpha}$[a]

	specific activity [nmol min^{-1} (nmol of P450)$^{-1}$]		K_m (μM)		V_{max} [nmol min^{-1} (nmol of P450)$^{-1}$]	
	−*b*5	+*b*5	−*b*5	+*b*5	−*b*5	+*b*5
P450coh	4.3 ± 0.3	9.4 ± 0.9	2.2	2.9	3.8	8.5
$P450_{7\alpha}$	17 ± 1	53 ± 3	2.9	0.5	8.8	24.2
$P450_{15\alpha}$	123 ± 6	84 ± 7				

[a] The specific activities of coumarin 7- and testosterone 7α- and 15α-hydroxylation in P450coh, $P450_{7\alpha}$, and $P450_{15\alpha}$, respectively, were measured in the presence (+*b*5) and absence (−*b*5) of *b*5. The coumarin 7-hydroxylation activity was measured at coumarin concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, and 100 μM using 10 pmol of P450coh. For the testosterone hydroxylation activities, the reaction mixtures contained 2 pmol of $P450_{7\alpha}$ or $P450_{15\alpha}$ and various concentrations of testosterone (2.0, 5.0, 10, 20, and 40 μM). The values of specific activity were obtained at 100 μM substrate concentration. Other assay conditions are described in the Materials and Methods.

RESULTS

Effects of b5 on the Hydroxylation Activities in P450coh, $P450_{7\alpha}$, and $P450_{15\alpha}$. The P450s were expressed in yeast cells and purified from yeast microsomes by ocytlamino-Sepharose 4B columns. The hydroxylation activity of the P450s were measured in a reconstitution system with and without the presence of *b*5 (Table I). The activities of P450coh and P4507α were stimulated by *b*5 2- and 3-fold, respectively. Conversely, $P450_{15\alpha}$ activity was decreased slightly in the presence of *b*5. Table I also shows the K_m and V_{max} values for P450coh and $P450_{7\alpha}$; the K_m value of P450coh activity was not changed by the presence of *b*5, while *b*5 decreased the K_m value of $P450_{7\alpha}$ activity 6-fold. We tested whether rat *b*5 also stimulated these activities, because Usanov et al. (1990) reported that only mouse *b*5 stimulated the coumarin 7-hydroxylation activity of P450coh. We found, however, that both rat and mouse *b*5 were equally effective in the stimulation by *b*5 (data not shown).

Stimulation Depending on Arg-129 of P450coh. P450coh and $P450_{15\alpha}$ differ only by 11 amino acids within their 494 residues (Lindberg & Negishi, 1989), yet the activity of the former P450 was stimulated by *b*5, while that of the latter P450 was not. Among these 11 residues, Arg-129 and His-320 in P450coh are positively charged amino acids. P45015α contains Ser and Tyr at positions 129 and 320, respectively. We, therefore, constructed and expressed P450coh mutants R129S and H320Y in which Arg-129 and His-320 of P450coh were substituted with Ser and Tyr, respectively, to study the effects of the mutations on the *b*5 dependent activity, because earlier studies (Tamburini et al., 1985; Stayton & Sligar, 1990) suggested that *b*5 interacts with P450 through its negatively charged amino acid(s). As shown in Figure 1, the coumarin 7-hydroxylation activity of mutant R129S was not enhanced at all by *b*5, although the activity of wild-type P450coh was stimulated 2-fold. The addition of *b*5 sharply increased the wild-type activity to its maximum level at a *b*5/P450 ratio of 1:1. The activity of mutant H320Y, on the other hand, was enhanced gradually as the *b*5/P450 ratio was increased and reached the 2-fold higher level at a ratio of 3:1. The results suggested, therefore, that Arg-129 plays a key

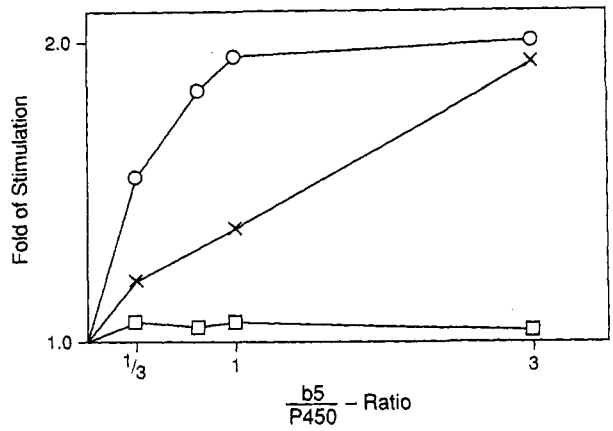

FIGURE 1: Effects of *b*5 on coumarin 7-hydroxylation activity of wild-type and mutant P450coh. Coumarin 7-hydroxylation activities of wild-type P450coh (○) and mutants R129S (□) and H320Y (×) were measured at various ratios of *b*5 and P450. For the experiments, the P450 content (10 pmol) was kept constant. We increased the amount of *b*5 to alter the *b*5/P450 ratio. Other assay condition are described in the Materials and Methods.

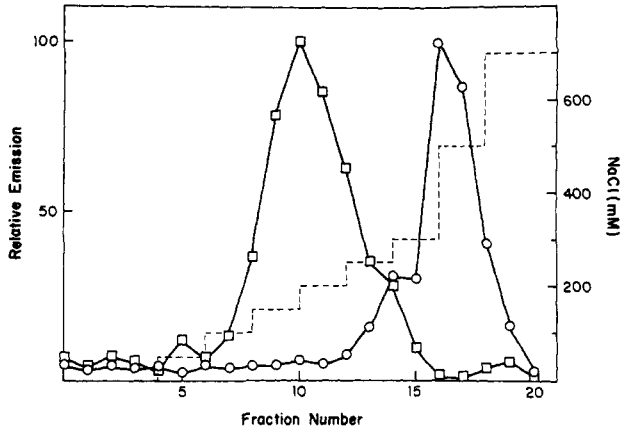

FIGURE 2: Affinity chromatography of wild-type and mutant P450coh on *b*5–Sepharose 4B. Wild-type P450coh (○) and mutant R129S (□) were bound to a *b*5–Sepharose 4B column (0.5 × 2 cm) and eluted by stepwise-increasing NaCl concentration (shown by broken line). One milliliter of eluate was collected for each fraction. Then, the coumarin 7-hydroxylation activities of these fractions were measured as described in the Materials and Methods. The values used in this figure are expressed by adjusting each activity value against the highest activity (fraction 10 of wild-type P450coh) as 100%.

role in the stimulation of P450coh activity by *b*5.

Binding of P450coh Depending on Arg-129. Affinity chromatography using a *b*5-conjugated Sepharose 4B column was performed to determine whether Arg-129 was involved in the binding of *b*5 to P450coh. Purified wild-type P450coh and mutant R129S were bound to the column and eluted with stepwise increases of the NaCl concentration (Figure 2). Mutant R129S was eluted from the column by 150–250 mM NaCl, while the wild-type P450coh was eluted at concentrations of 250–500 mM. The other mutants H320Y and F209G (data not shown) were eluted from the column like the wild-type P450coh. The wild-type $P450_{15\alpha}$, on the other hand, mimicked the elution pattern of the mutant R129S. Rat *b*5 produced type II substrate-induced spectra with both wild type and R129S (not shown). The spectra were obtained at seven different *b*5 concentrations between 20 and 1200 μM and were used for the basis to estimate the K_a values. The K_a for *b*5 binding to wild-type P450coh was 2.5 μM, approximately 6 times lower than the 400 μM of mutant R129S. The mutation of Arg-129 to Ser in P450coh, therefore, appeared to decrease its binding affinity to *b*5.

Effect of the Identity of Residue 209 on b5-Dependent Stimulation. The identity of residue 209 also altered *b*5-dependent stimulation of P450 activities (Figure 3A). Coumarin 7-hydroxylation activity was stimulated 2-fold by *b*5 only when residue 209 was Phe. A slight but significant stimulation by *b*5 was observed with mutants F209L, F209G, F209N, and F209S in which Phe-209 was substituted by Leu, Gly, Asn, and Ser, respectively. Moreover, the enzyme activity was not enhanced by *b*5 when Val (F209V), Ala (F209A), or Lys (F209K) substituted residue 209 in P450coh (Figure 3A). Furthermore, we measured the activity of each mutant at the various b5/P450 ratios up to 3:1 (data not shown) and found that the *b*5 stimulation in each mutant did not depend on the ratio. It appeared, therefore, that the mutations of residue 209 did not alter the surface structure of P450coh and the *b*5–P450 interaction. In addition to coumarin 7-hydroxylation activity, P450coh also catalyzed testosterone 7α- and 15α-hydroxylation activities, albeit at 50- and 100-fold lower levels when they were compared with the activities of wild-type $P450_{7\alpha}$ and $P450_{15\alpha}$, respectively (Iwasaki et al., 1991, 1992). As seen in Figure 3, panels B and C, *b*5 stimulated these testosterone hydroxylation activities differently depending on the type of residue 209. 7α-Hydroxylation activity of P450coh was increased approximately 2-fold by *b*5 when residue 209 was Phe, Asn or Ser. 15α-Hydroxylation activity, on the other hand, was increased 2–3-fold by *b*5 when residue 209 was Phe, Ser, or Lys and to a lesser extent when residue 209 was Asn. Interestingly, when residue 209 was Leu, the 15α-hydroxylation activity was decreased by *b*5.

Effects of the Type of Residue 209 and b5 on the Rate of Hydrogen Peroxide Formation. The rate of hydrogen peroxide formation by P450coh was altered depending on the identity of residue 209. Moreover, *b*5 decreased the hydrogen peroxide formation depending on the types of residue 209 and substrate (Table II). When coumarin was the substrate, placing a negatively-charged amino acid (Asp, for example) at position 209 increased the hydrogen peroxide formation of P450coh approximately 3-fold compared with the wild-type P450coh, while smaller hydrophobic amino acids such as Ala or positively-charged Lys at this position decreased it approximately 4-fold. The same mutants exhibited similar altered hydrogen peroxide formation when testosterone was the substrate, except that F209K did not decrease the formation. The addition of *b*5 decreased the hydrogen peroxide formation of wild-type P450coh (Phe-209) and mutant F209D when coumarin was the substrate. Conversely, the mutants F209N and F209D decreased their hydrogen peroxide formation when testosterone was substrate. To a lesser degree, the mutant F209L also decreased the formation of hydrogen peroxide in the presence of *b*5.

DISCUSSION

We demonstrate here that Arg-129 in P450coh serves as a major site for the *b*5–P450 interaction. This residue is essential for the *b*5-dependent stimulation of coumarin 7-hydroxylation activity in P450coh. Consistent with the critical role of Arg-129 in the stimulation of P450 activity, $P450_{7\alpha}$ also contains the corresponding Arg and *b*5 increases the testosterone 7α-hydroxylation activity. The addition of *b*5, on the other hand, slightly decreases the testosterone 15α-hydroxylation activity of $P450_{15\alpha}$ in which Ser substitutes residue 129. In addition to Arg-129, His-320 also influences the *b*5-dependent stimulation of P450coh activity. While His-320 is not essential for the stimulation, the mutations of this residue does alter the mode of stimulation. The activity of

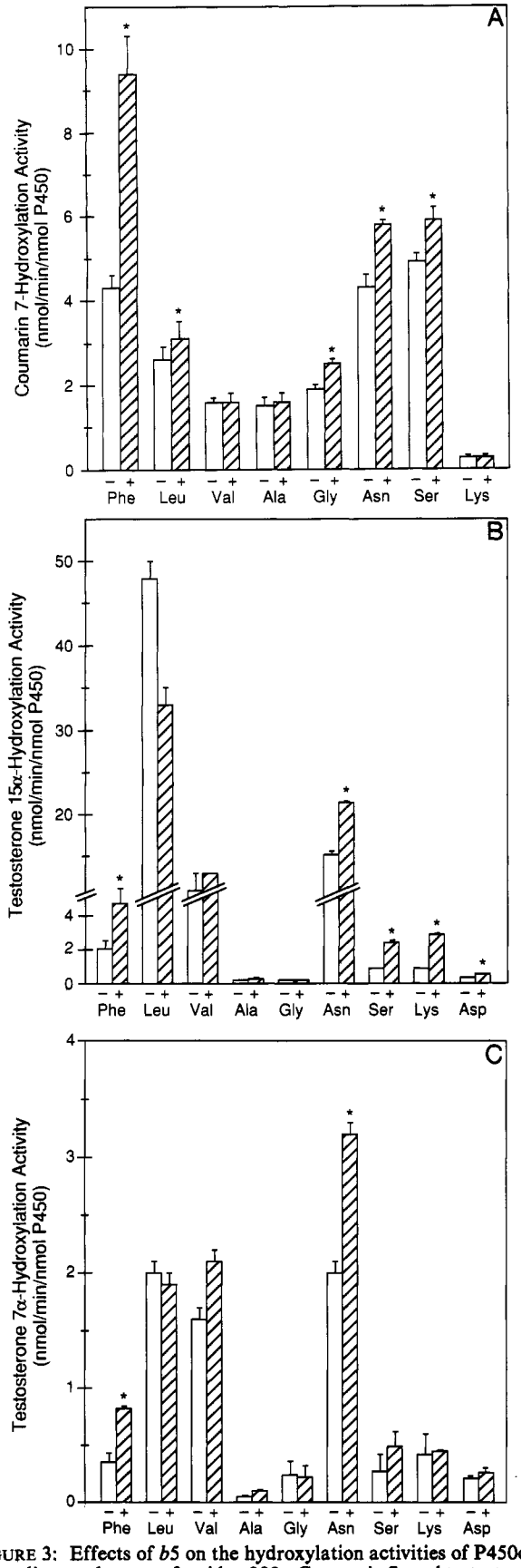

FIGURE 3: Effects of *b5* on the hydroxylation activities of P450coh depending on the type of residue 209. Coumarin 7- and testosterone 7α- and 15α-hydroxylation activities of wild-type and mutant P450coh were determined with (hatched bars) and without (open bars) the presence of *b5*; 20 pmol of *b5* was added to the reaction mixture containing 10 pmol of P450. Other conditions of the reconstitution assay are described in the Materials and Methods. The mutants P450 were constructed by substituting Phe-209 in P450coh with various amino acids, which are indicated under each pair of bars.

Table II: Effect of *b5* on Hydrogen Peroxide Formation of Wild-Type P450coh and Its Residue 209 Substituted Mutants[a]

	hydrogen peroxide formation [nmol min^{-1} (nmol of P450)$^{-1}$]			
	coumarin		testosterone	
	−*b5*	+*b5*	−*b5*	+*b5*
wild type, Phe-209	0.83 ± 0.8	0.58 ± 0.6[b]	0.64 ± 0.19	0.55 ± 0.20
F209L	0.43 ± 0.07	0.34 ± 0.05	0.89 ± 0.09	0.81 ± 0.08[b]
F209A	0.30 ± 0.09	0.31 ± 0.06	0.46 ± 0.11	0.48 ± 0.08
F209N	1.07 ± 0.23	0.96 ± 0.11	1.47 ± 0.06	1.20 ± 0.11[b]
F209K	0.23 ± 0.03	0.24 ± 0.06	0.77 ± 0.14	0.84 ± 0.19
F209D	2.10 ± 0.36	1.64 ± 0.31[b]	2.21 ± 0.03	1.83 ± 0.02[b]

[a] Hydrogen peroxide formation was measured in the same incubation conditions as used for the hydroxylation activities in Figure 3. The values in this table were the averages of four different measurements and were analyzed by the paired Student's *T*-test to obtain *P* values. [b] Differences obtained with *b5* are statistically significant ($P < 0.05$).

mutant H320Y increases gradually and reaches a maximum level at a *b5*/P450 ratio of 3:1. Arg-129 and His-320 are mapped close to the different substrate recognition sites of P450coh. Recently, Gotoh (1992) mapped six putative SRSs (substrate recognition sites) in the mammalian 2A P450s by aligning their amino acid sequences to the bacterial P450cam in which the substrate binding sites are well defined (Poulos et al., 1987). According to Gotoh's map, Arg-129 and His-320 are located close to SRS1 and SRS4, respectively. Although their mechanisms are unknown, the topologies of these residues relative to the SRSs may determine the differences in the *b5*-dependent stimulation of P450coh activity.

Arg-129, a key residue interacting with *b5*, is the second Arg in the phosphorylation motif Arg-Arg-X-Ser by cAMP-dependent protein kinase (PKA). Our current results, therefore, support a previous suggestion by Schenkman and his associates that a *b5*-binding site is nested in the phosphorylation site (Jansson et al., 1987; Epstein et al., 1989). The *b5*-binding and PKA-dependent phosphorylation sites overlap in these P450s. Numerous works have reported that the P450s can be phosphorylated in vivo as well as in vitro (Eliasson et al., 1990; Jansson et al., 1987, 1990; Koch & Waxman, 1989; Pyerin & Taniguchi, 1989). The phosphorylated P450s, however, are denatured proteins and rapidly degrade in vivo. It is still in question whether the denaturation of P450 precedes its phosphorylation or the phosphorylation results in denaturation of P450 (Pyerin et al., 1983; Jansson et al., 1987). Regardless of which event occurs first, however, a phosphorylated P450 does not seem to be an active enzyme. It appears, therefore, that the phosphorylation should play no role in the *b5*-dependent stimulation. The physiological significance of the overlap of phosphorylation and *b5*-binding sites remains to be understood.

Pompon and Coon (1984) and Pompon (1987) have suggested that the transfer of the second electron from *b5* increases the product formation of P450 by decreasing the hydrogen peroxide formation. Residue 209 resides close to the sixth ligand of heme in P450coh (Iwasaki et al., 1992) and plays a key role in determining the substrate and product specificities as well as K_m and V_{max} of the P450 (Lindberg & Negishi, 1989; Juvonen et al., 1992). It is reasonable, therefore, to assume that *b5* alters differentially the hydroxylation activity and hydrogen peroxide formation depending on the identity of residue 209. Considering a model proposed by Pompon (1982) and our current results, residue 209 seems to regulate the one-electron reduction of the ferrous dioxygen

complex P450[$Fe^{II}O_2$]. When the types of residue 209 of P450, the substrate used, and the product formed are in the proper combinations, *b*5 can increase the product formation by altering the rate of one-electron reduction of P450[$Fe^{II}O_2$] and the formation of hydrogen peroxide. When residue 209 is Phe, for example, *b*5 increases the coumarin 7-hydroxylation activity and decreases the hydrogen peroxide formation. Similarly, *b*5 increases the testosterone hydroxylation activities of mutant F209N and decreases its hydrogen peroxide formation. However, this correlation between the hydroxylation activity and the hydrogen peroxide formation does not always occur. *b*5, for example, stimulates testosterone hydroxylation activities of wild-type P450coh and coumarin 7-hydroxylation activity of mutants F209K and F209N. Their hydrogen peroxide formations, however, are not decreased. Moreover, *b*5 decreases both testosterone 15α-hydroxylation activity and hydrogen peroxide formation by mutant F209L.

In conclusion, Arg-129 of P450coh appears to serve as the major *b*5-binding site. This amino acid is essential for the *b*5-dependent alteration of P450coh activity regardless of the types of substrate used and product formed. The effect of residue 209, on the other hand, depends on its identity and also on the types of substrate and product. As an amino acid near the sixth ligand of heme in P450coh, residue 209 appears to regulate the efficiency of product formation.

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Registry No. Arg, 74-79-3; Phe, 63-91-2; Asn, 70-47-3; Ser, 56-45-1; Lys, 56-87-1; Asp, 56-84-8; Ala, 56-41-7; Leu, 61-90-5; *b*5, 9035-39-6; P450, 9035-51-2; coh, 39401-02-0; H_2O_2, 7722-84-1; 7α, 39346-35-5; 15α, 70699-33-1.