Site-directed mutagenesis of mouse steroid 7α-hydroxylase (cytochrome *P*-450$_{7\alpha}$): role of residue-209 in determining steroid–cytochrome *P*-450 interaction

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We have cloned a cDNA encoding mouse steroid 7α-hydroxylase P450$_{7\alpha}$ (cytochrome *P*-450$_{7\alpha}$) and expressed it in *Saccharomyces cerevisiae*. Mouse P450$_{7\alpha}$ is 70% identical in its amino acid sequence with the mouse steroid 15α-hydroxylase P450$_{15\alpha}$ (2A4). The Leu at position 209 of P450$_{15\alpha}$ is the most important residue to determine the steroid hydroxylase activity of the P450 [Lindberg and Negishi (1989) Nature (London) **339**, 632–634]. The P450$_{7\alpha}$ contains Asn at the position corresponding to the Leu-209 of P450$_{15\alpha}$, although both P450s hydroxylate testosterone. The CO-reduced P450$_{7\alpha}$ complex is unstable, so that it is quickly converted into the inactive P420, whereas the P450$_{15\alpha}$ is very stable. The P450$_{7\alpha}$, however, is stabilized either by addition of testosterone or by a mutation of Asn-209 to Leu. The mutant P450$_{7\alpha}$ displays a 17-fold lower $V_{max.}$ value than the wild-type enzyme. Unexpectedly, it also has 3-fold lower K_m and K_d values. Residue 209 in P450$_{7\alpha}$, therefore, appears to be located at a critical site of the haem-substrate-binding pocket. Corticosterone inhibits the testosterone 7α-hydroxylase activity of the wild-type P450$_{7\alpha}$, whereas it does not inhibit the mutant P450$_{7\alpha}$. Conversely, the P450$_{15\alpha}$ activity becomes inhibited by corticosterone upon the replacement of Leu-209 by Asn. In addition, this mutation increases the corticosterone 15α-hydroxylase activity of P450$_{15\alpha}$ at least 20-fold. Whereas the inhibition by corticosterone depends on the presence of Asn at position 209, deoxycorticosterone inhibits the activities of the P450s regardless of the type of residue at 209. The results indicate, therefore, that the identity of residue 209 determines the affinity as well as specificity of steroid binding to both P450$_{7\alpha}$ and P450$_{15\alpha}$.

INTRODUCTION

Cytochrome *P*-450s (P450s), as a large family of structurally related enzymes, serve as terminal mono-oxygenases of the membrane-bound electron-transport system. Although, collectively, P450s display a broad range of substrate and product specificities, individually they often are characterized by a high degree of specificity. It is therefore essential to identify the substrate-binding sites of mammalian P450s in order to understand the structural basis of the paradoxical nature of the enzymes' specificities. This is a major interest in P450 research.

Mouse steroid 15α-hydroxylase P450$_{15\alpha}$ (2A4) and coumarin 7-hydroxylase P450coh (2A5) differ only by 11 residues within their 494 amino acids (Squires and Negishi, 1988; Lindberg et al., 1989; Negishi et al., 1989). Our site-directed mutagenesis study shows that residue 209 is the most critical amino acid in determining the substrate specificity of the P450s (Lindberg and Negishi, 1989), because the identity of residue-209 alters the substrate specificity of P450coh from coumarin to steroid hydroxylase activity. In order to examine the topology of residue-209 in the P450s, we constructed and expressed a series of mutant P450s in which residue-209 of P450$_{15\alpha}$ and P450coh is replaced by various amino acids (Iwasaki et al., 1991; Juvonen et al., 1991). The spin-state alteration by the mutations of these P450s indicates that residue 209 residues cose to the sixth axial position of the haem (Iwasaki et al., 1991). In addition, the type of residue 209 alters the K_m and K_d values of the mutant P450s 200-fold (Juvonen et al., 1991). Our findings, therefore, depend on the close localization of the residue 209 to the haem and the substrate-binding site. However, a 'homology' alignment of the mammalian P450s to the bacterial P450cam suggests that the residue 209 of P450$_{15\alpha}$ and P450coh would be located in the region corresponding to helix E in the P450cam (Nelson and Strobel, 1989). This helix is on the surface of P450cam molecule and is far from the haem-substrate pocket (Poulos, 1991). The topology of residue 209 in the mouse P450s, therefore, remains to be determined.

In the present study, we cloned a cDNA for mouse steroid 7α-hydroxylase P450$_{7\alpha}$, which is closely related to P450$_{15\alpha}$ and P450coh. Using the P450$_{7\alpha}$ and its mutants we further examine the role of the residue 209 in determining the specificity of the binding of steroid to the P450s.

EXPERIMENTAL

cDNA cloning

A cDNA library from AKR/J mice was constructed with the λZAP vector system (Stratagene) and screened with a ^{32}P-labelled P450$_{15\alpha}$ cDNA (Lindberg et al., 1989) as a probe. The weakly hybridized recombinant phages were purified and expressed in COS 1 cells to select a P450$_{7\alpha}$ cDNA. Finally, the cDNA was subcloned into M13 vector and sequenced using the dideoxy-

Abbreviations used: P450, cytochrome *P*-450; SRS, substrate-recognition sites.

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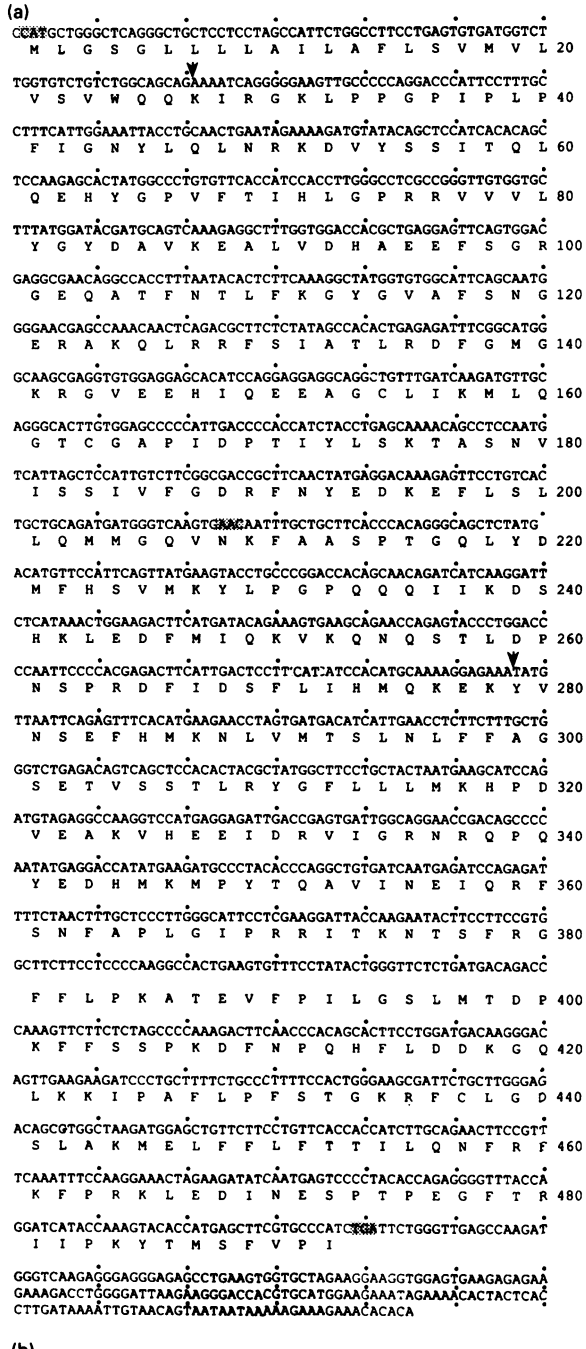
The nucleotide sequence reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number LO6463.



nucleotide termination reaction (Sanger et al., 1980; Biggin et al., 1983; Tabor and Richardson, 1987), [α-^{35}S]dATP, Sequenase (United States Biochemicals) and specific primers. We used programs in the University of Wisconsin Genetics Computer Group package BESTFIT to analyse the nucleotide sequence.

Site-directed mutagenesis and expression in yeast cells

We first created the 5′ and 3′-terminal *Hin*dIII sites by the amplification of the $P450_{7\alpha}$ cDNA using the 5′-primer (5′-CGAAGCTTAAAAAAATGCTGGGCTCAGGGC) and the 3′-primer (5′-CGAAGCTTTCCATGCACGGTGGTCCCTT) and PCR kit (Cetus–Perkin–Elmer). For the mutagenesis, the amplified cDNAs were digested by *Hin*dIII and ligated to pSELECT vector (Promega). The following oligonucleotides were used to mutate each amino acid at positions 207, 209 and 210 in the $P450_{7\alpha}$. 5′-ATGATGGGTGGCGTGAACAAA, 5′-GGTCAAGTGCTCAAATTTGCT, 5′-CAAGTGAACCAATTTGCTGCT, respectively. The underlinings show the codons mutated. Then the mutated cDNAs were inserted into the *Hin*dIII site of the yeast expression vector pAAH5 and transfected into *Saccharomyces cerevisiae* AH22 using the LiCl method (Oeda et al., 1985; Iwasaki et al., 1991). Finally we partially purified, using amino-octyl-Sepharose 4B and hydroxyapatite columns, the wild-type and mutated P450s from the solubilized yeast microsomes using the previously published method (Iwasaki et al., 1991; Juvonen et al., 1991).

Analytical methods

P450 and protein contents were determined by the methods of Omura and Sato (1964) and Bradford (1976) respectively. The previously reported procedure (Harada and Negishi, 1988) was used to measure the steroid 7α- and 15α-hydroxylase activities of the recombinant P450s. Western blots were done by the method of Domin et al. (1984).

Chemicals

We purchased [4-^{14}C]testosterone (52 mCi/mmol) and [4-^{14}C]-corticosterone (52 mCi/mmol) from Amersham International. Coumarin, testosterone, corticosterone, deoxycorticosterone, progesterone, and 11β-hydroxycorticosterone were obtained from Sigma and silica-gel plates from EM Laboratory (Elmford, NY, U.S.A.) All other chemicals used were of the highest quality commercially available.

RESULTS

Nucleotide and deduced amino acid sequence

Mouse $P450_{7\alpha}$ consists of 492 amino acid residues and has two amino acid deletions which correspond to the residues 27 and 279 of the $P450_{15\alpha}$ and P450coh (Figure 1a). The $P450_{7\alpha}$ is 70 and 71% identical in its amino acid sequence with the $P450_{15\alpha}$ and P450coh respectively. Mouse $P450_{7\alpha}$, therefore, is the third member of the mouse 2A subfamily according to current nomenclature (Nebert et al., 1991). As expected, mouse $P450_{7\alpha}$

Figure 1 Nucleotide and deduced amino acid sequence of $P450_{7\alpha}$ cDNA

(**a**) The initiation and termination codons are boxed. The Asp corresponding to Leu-209 in mouse $P450_{15\alpha}$ is boxed also. The positions of two amino acids which are deleted in the $P450_{7\alpha}$ are indicated by the arrows. (**b**) A 'homology' alignment of the amino acid sequence of $P450_{7\alpha}$ to the $P450_{15\alpha}$. The Leu-209 of $P450_{15\alpha}$ and the corresponding Asn of $P450_{7\alpha}$ are boxed.

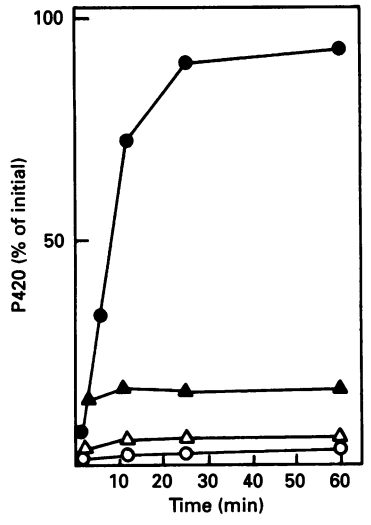


Figure 2 Stabilization by testosterone and mutation of Asn-209 of CO-reduced $P450_{7\alpha}$ complex

The CO-difference spectra of the reduced $P450_{7\alpha}$ and mutant S_7209(Asn-Leu) were obtained in the presence (open circles and triangles for the wild-type and mutant respectively) and the absence (the closed symbols) of 100 μM testosterone at each time point. The relative amounts of the P420s are expressed as their percentages to the initial amounts of the P450s in the samples. To obtain the spectra, we used 0.2 μM of each P450 in a solution of 100 mM potassium phosphate buffer, pH 7.25 containing 20% glycerol, 0.04% sodium cholate, 0.02% Emulgen 913, 1 mM EDTA and 1 mM dithiothreitol.

Table 1 Enzyme-kinetic properties of wild-type and mutants $P450_{7\alpha}$

The testosterone 7α-hydroxylase activity of the P450s was reconstituted by using a previously described procedure (Harada and Negishi, 1988). The reconstitution system contained P450 (20 pmol), NADPH:cytochrome P450 reductase (30 pmol), NADPH (0.5 mM), $MgCl_2$ (5 mM), didodecylphosphatidylcholine (6.0 nmol) and [4-^{14}C]testosterone (2, 10, 20, 40, 100 and 400 μM; 26 μCi/mmol) in 0.5 ml of 50 mM Tris/HCl buffer, pH 7.5. The K_m and $V_{max.}$ values were obtained by the Lineweaver–Burk method. The substrate-induced difference spectra of the P450s (0.2 μM in 100 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol) were obtained in the presence of various testosterone concentrations (0.5, 1.0, 4.0, 20, 140, and 340 μM). The K_d values were calculated from the testosterone-dissociation constant (K_d) as described previously (Jefcoate, 1978; Lange et al., 1988).

	K_m (μM)	K_d (μM)	$V_{max.}$ (nmol/min per nmol of P450)
Wild-type $P450_{7\alpha}$	11.8	6.5	28.0
S_7207(Gln-Gly)	5.7	2.9	17.0
S_7209(Asn-Leu)	3.6	1.8	1.6
S_7209(Lys-Gln)	10.8	6.5	35.0

exhibits the higher amino acid sequence identity (88.4%) with the rat testosterone 7α-hydroxylase $P450_{7\alpha}$ (2A1) (Matsunaga et al., 1988) than with the other mouse subfamily members. The Leu at position 209 in the $P450_{15\alpha}$ plays the critical role in determining the steroid hydroxylase activity of the P450 (Lindberg and Negishi, 1989). Mouse $P450_{7\alpha}$, however, has Asn at position 209. In fact, the region around residue 209 is one of the most divergent sequences between the two mouse steroid hydroxylases P450 (Figure 1b). To examine the functions of the regional amino acids we mutated Gln-207, Asn-209 and Lys-210 in the $P450_{7\alpha}$ to the corresponding amino acid of the $P450_{15\alpha}$. These mutants, which were designated S_7207(Gln-Gly). S_7209(Asn-Leu) and S_7210(Lys-Gln) respectively, were expressed in yeast cells and their spectral and catalytic properties studied. Because the residue at position 27 is deleted in the $P450_{7\alpha}$, the Asn is actually positioned at 208 in the P450. Here we use, however, the amino acid numbering of the $P450_{15\alpha}$ to represent the amino acids of the $P450_{7\alpha}$ for convenience.

Blue shift of the Soret peak and stabilization of the CO-reduced $P450_{7\alpha}$ complex

The Soret peak of the CO difference spectrum of the reduced wild-type $P450_{7\alpha}$ appears at 452 nm, while a mutation S_7209(Asn-Leu) shifts the position of Soret peak to 448 nm (results not shown). Similarly, the mutant S_7207(Gln-Gly) displays the Soret peak at 448 nm. The mutation S_7210(Lys-Gln), on the other hand, maintains the Soret peak at 452 nm. The absolute spectra of the oxidized forms of wild-type and mutant $P450_{7\alpha}$ indicate that they are all low-spin P450s. The CO-reduced $P450_{7\alpha}$ complex is unstable, so that it is rapidly converted into the inactive P420. The half-life of this spectral conversion is approx. 10 min (Figure 2). In the presence of testosterone, however, the $P450_{7\alpha}$ is not converted into the P420 within the 60 min incubation. The mutant S_7209(Asn-Leu), on the other hand, is a stable P450, regardless of the presence of testosterone (Figure 2). The $P450_{7\alpha}$, therefore, can be stabilized either by the presence of testosterone or by the mutation of Asn-209 to Leu.

Alteration of K_m, $V_{max.}$ and K_d values

We first measured the K_m and $V_{max.}$ values for the testosterone 7α-hydroxylase activity of wild-type $P450_{7\alpha}$ and its mutants. As expected, the mutation S_7209(Asn-Leu) decreases the $V_{max.}$ value 17-fold compared with that seen with the wild-type $P450_{7\alpha}$. Unexpectedly, the mutation also decreases the K_m value approx. 3-fold (Table 1). We then measured the K_d values of the wild-type and mutant $P450_{7\alpha}$ based on their Type I spectra with testosterone (results not shown). Consistent with the decrease of the K_m value, the mutation S_7209(Asn-Leu) also decreases the K_d value for testosterone binding approx. 3-fold. Other mutations, S_7207(Gln-Gly) and S_7210(Lys-Gln), do not change their catalytic properties (K_m, $V_{max.}$ and K_d values) as much as S_7209(Asn-Leu) does. The $P450_{7\alpha}$, therefore, appears to have the higher testosterone-binding affinity when residue 209 is mutated to Leu, although the reaction rate is decreased considerably.

Differential inhibition by 11β-hydroxy- and 11-deoxy-steroids

We first used corticosterone and deoxycorticosterone to inhibit the testosterone hydroxylase activity of wild-types $P450_{7\alpha}$ and $P450_{15\alpha}$ and their mutants (Figure 3). Corticosterone, a 11β-hydroxysteroid, inhibits the activity of the wild-type $P450_{7\alpha}$ but not of the mutant S_7209(Asn-Leu). The steroid at 300–500 μM, for example, completely inhibits the activity of the wild-type $P450_{7\alpha}$, while it does not affect the activity of mutant S_7209(Asn-Leu). Conversely, corticosterone inhibits the activity of mutant $P450_{15\alpha}$ $S_{15}209$(Leu-Asn), but not of wild-type $P450_{15\alpha}$. The inhibition of corticosterone, therefore, depends on the presence of Asn at position 209 in the P450s. Deoxycorticosterone, on the other hand, inhibits the both wild-type and mutant $P450_{7\alpha}$ and $P450_{15\alpha}$ irrespective of the presence of Asn at position 209. To further confirm the findings, we tested progesterone and 11β-hydroxyprogesterone to inhibit the testosterone hydroxylase activity of wild-type and mutant P450s (Table 2). Like

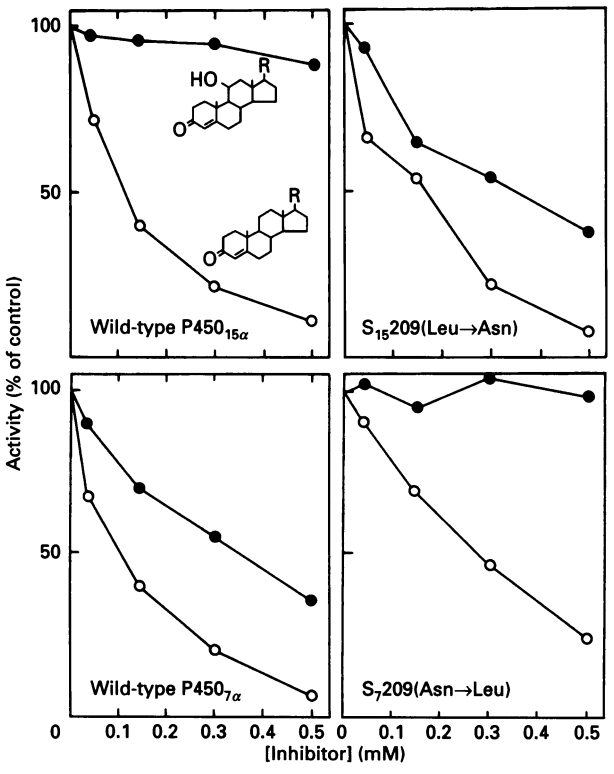


Figure 3 Inhibition by corticosterone and deoxycorticosterone of the testosterone 5α-hydroxylase activity of the wild-types P450$_{7\alpha}$ and P450$_{15\alpha}$ and their mutants

Testosterone 7α and 15α-hydroxylase activities of wild-type and mutant P450$_{7\alpha}$ and P450$_{15\alpha}$ were reconstituted in the presence of various concentrations of corticosterone (closed circles) or deoxycorticosterone (open circles). The experiments wee repeated twice and averaged to provide the values in this Figure. This Figure also includes the structures of the steroid inhibitors: R = —$COCH_2OH$.

Table 2 Inhibition, by 11β-hydroxysteroids and 11-deoxysteroids, of the testosterone hydroxylase activities of wild-type and mutant P450s

The testosterone 7α- and 15α-hydroxylase activities of the P450s were reconstituted in the presence and the absence of 500 μM steroid inhibitor. The composition of the reconstitution system is described in the legend for Table 1, except that the 50 μM [4-^{14}C]testosterone was used as the substrate. The residual activities are presented as percentages of the activities without the inhibitor.

	Residual activity (%)				
Inhibitor	P450$_{7\alpha}$	S$_7$207-(Gln-Gly)	S$_7$209-(Asn-Leu)	S$_7$210-(Lys-Gln)	P450$_{15\alpha}$
Progesterone	5.9	13.3	15.5	8.6	17.1
1β-hydroxy-progesterone	16.8	27.2	62.4	28.9	57.6
Deoxy-corticosterone	7.8	9.4	25.6	16.0	15.1
Corticosterone	36.0	28.0	98.9	35.2	84.7
Comain	93.5	67.5	59.3	83.1	16.0

deoxycorticosterone, progesterone efficiently inhibits the hydroxylase activity of each P450 regardless of the type of residue at position 209. Consistent with the fact that the inhibition by corticosterone depends on Asn-209 of the P450 and the 11β-hydroxy of steroid molecule, 11β-hydroxyprogesterone inhibits more efficiently the activity of the P450s with Asn at position 209

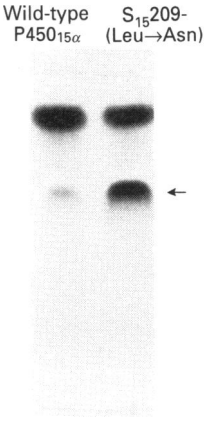


Figure 4 Corticosterone 15α-hydroxylase activity of the wild-type P450$_{15\alpha}$ and its mutant S$_{15}$209(Leu-Asn)

The corticosterone 15α-hydroxylase activity of the P450s was reconstituted as described in Table 1, except that [4-^{14}C]corticosterone (5 μM) was used as the substrate. Corticosterone metabolite was extracted and analysed by t.l.c. using the conditions previously reported (Harada and Negishi, 1988). The t.l.c. plate was exposed to a Kodak X-ray film and the radioactivity of the metabolite was measured by scintillation counting. The structure of the metabolite has been determined to be the 15α-hydroxycorticosterone (Iwasaki et al., 1993).

(Table 2). More than 80% of the P450$_{7\alpha}$ activity is inhibited by 11β-progesterone, while this steroid inhibits only less than 40% of the activity of mutant S$_7$209(Asn-Leu). We conclude, therefore, that the inhibition of the testosterone hydroxylase activity by the 11β-hydroxysteroids depends on the presence of Asn at position 209 in the P450s. Interestingly, coumarin inhibits the activities of mutant P450$_{7\alpha}$ S$_7$209(Asn-Leu) and wild-type P450$_{15\alpha}$, although it does not inhibit the activity of wild-type P450$_{7\alpha}$ at all (Table 2). The inhibition by coumarin, therefore, depends on the presence of Leu at position 209, which agrees with our previous findings that the presence of a hydrophobic amino acid at position 209 is critical for the binding and metabolism of coumarin by the P450$_{15\alpha}$ and P450coh (Lindberg and Negishi, 1989; Iwasaki et al., 1991; Juvonen et al., 1991).

Alteration of steroid-substrate specificity of P450$_{15\alpha}$ from testosterone to corticosterone by mutation of Leu-209 to Asn

The P450$_{15\alpha}$ specifically catalyses the 15α-hydroxylation of Δ^4-3-ketone steroids, including testosterone, progesterone and androstenedione, but it does not metabolize corticosterone (Harada and Negishi, 1988; Lindberg et al., 1989). Moreover, previous site-directed-mutagenesis studies showed that a mutation of Leu-209 to Asn decreases the testosterone 15α-hydroxylase activity 10-fold compared with the wild-type P450$_{15\alpha}$ (Iwasaki et al., 1991). To examine whether a mutation of Leu-209 to Asn in P450$_{15\alpha}$ affects the steroid-substrate-specificity of the P450$_{15\alpha}$, we measured the corticosterone hydroxylase activity of mutant S$_{15}$209(Leu-Asn). The mutation S$_{15}$209(Leu-Asn) resulted in the 20-fold increase of the activity of P450$_{15\alpha}$ (Figure 4). The P450$_{7\alpha}$, on the other hand, did not catalyse corticosterone hydroxylase activity, although it has Asn at position 209 and exhibits a higher binding affinity to the 11β-hydroxysteroids than to the 11-deoxysteroids.

DISCUSSION

We now consider steroid 7α-hydroxylase P450$_{7\alpha}$ to be the third member of the mouse P4502A subfamily. As in the rat 2A subfamily, the mouse 2A subfamily consists of the three members: steroid 15α-hydroxylase P450$_{15\alpha}$ (2A4) and coumarin 7-

hydroxylase P450coh (2A5), in addition to the $P450_{7\alpha}$. We have previously suggested that rat CYP2A3, Mouse *Cyp*2A5 and human CPY2A6 are orthologous, because they conserve very well their coumarin 7-hydroxylase activities and structures (Negishi et al., 1992). Similarly, the high structural and functional conservation suggests that mouse and rat steroid 7α-hydroxylases are also encoded by orthologous P450 genes.

Residue 209 of $P450_{15\alpha}$ and P450coh appear to reside close the sixth ligand of the haem in the P450s, because the spin equilibria of the P450s are shifted more toward low-spin states as the residue becomes smaller in its size and/or more hydrophilic (Iwasaki et al., 1991). A similar spin alteration, depending on the identity of residue 209, however, is not observed in $P450_{7\alpha}$. Instead, a substitution of Asn-209 with Leu shifts the position of P450's Soret peak in the CO-reduced difference spectrum from 452 to 448 nm. Moreover, this mutation stabilizes the $P450_{7\alpha}$ from conversion into the inactive P420. In addition to these spectral changes, the mutation of Asn-209 in $P450_{7\alpha}$ results in the decreases of the K_m and $V_{max.}$ values for the testosterone 7α-hydroxylase activity and also of the K_d value for the testosterone binding. The present results, therefore, indicate that residue 209 of $P450_{7\alpha}$ is also located close to a substrate-binding site and to the haem of P450.

According to a predicted structure of mammalian P450s by aligning their amino acid sequences with that of bacterial P450cam (Nelson and Strobel, 1989), residue 209 of $P450_{15\alpha}$ would be located in a region corresponding to helix E far from the substrate-haem pocket (Poulos, 1991). Recently, Gotoh (1992) has proposed a new sequence alignment of the 2A subfamily members to the P450cam and identified the six putative substrate-recognition sites (SRSs) in mammalian P450s. Unlike Nelson and Strobel's (1989) alignment, Gotoh (1992) maps residue 209 in SRS-2, which is located in the F–G interhelical region of P450cam. Also, our recent alignment of P450coh to P450cam is very similar to that proposed by Gotoh (1992) (Iwasaki et al., 1993). The Gotoh (1992) alignment can very well explain the recent site-directed-mutagenesis studies (Lindberg and Negishi, 1989; Imai and Nakamura, 1989; Furuya et al., 1989; Aoyama et al., 1989; Matsunaga et al., 1990; Graham-Lorence et al., 1991; Kronbach and Johnson, 1991). It appears, therefore, that the basic structure of the substrate-haem-binding pocket is very well conserved in the bacterial and mammalian P450s. Moreover, the amino acids corresponding to residue 209 may play an important role in determining the catalytic specificity of many other mammalian P450s.

Different steroids behave differently as substrates and inhibitors depending on the nature of residue 209 in the $P450_{7\alpha}$ and $P450_{15\alpha}$. The 11β-hydroxysteroids, including corticosterone and 11β-hydroxyprogesterone, are good inhibitors of testosterone hydroxylase activity of those P450s with Asn at position 209. For the 11β-hydroxysteroids to be inhibitors, therefore, depends on the presence of Asn at position 209. The 11-deoxysteroids, such as deoxycorticosterone and progesterone, on the other hand, inhibit the P450 activities irrespective of the type of residue 209. Moreover, the Leu-209-to-Asn mutation of $P450_{15\alpha}$ results in the 20-fold increase of corticosterone 15α-hydroxylase activity of the P450, while the mutation reciprocally decreases the testosterone 15α-hydroxylase activity. As shown by the lower K_d value, the mutation also increases the binding affinity of $P450_{7\alpha}$ to testosterone. These results suggest that the type of substituents at C11 of the steroids plays an important role in the steroid binding to the P450s. Moreover, despite their diversity, the amino acid region around the residue 209 of $P450_{7\alpha}$ and $P450_{15\alpha}$ retain a similar interaction with the steroid molecule. In conclusion, the substrate-binding specificity and affinity of steroid to the active site of the $P450_{7\alpha}$ and $P450_{15\alpha}$, therefore, can be altered by the combination of the nature of the residue at position 209 in the P450s and the substituent at C-11 of the steroid molecule.

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REFERENCES

Aoyama, T., Korzekwa, K., Nagata, K., Adesnik, M., Reiss, A., Lapenson, D. P., Gillette, J., Gelboin, H., Waxman, D. J. and Gonzalez, F. J. (1989) J. Biol. Chem. **264**, 21327–21333

Biggin, W. D., Gibson, T. J. and Hong, G. F. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3963–3965

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Domin, B. A., Serabjit-Singh, C. J. and Philpot, R. M. (1984) Anal. Biochem. **136**, 390–396

Gotoh, O. (1992) J. Biol. Chem. **267**, 83–89

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

Graham-Lorence, S., Khail, M. W., Lorence, M. C., Mendelson, C. R. and Simpson, E. R. (1991) J. Biol. Chem. **266**, 11939–11946

Harada, N. and Negishi, M. (1988) Biochem. Pharmacol. **34**, 4778–4780

Imai, Y. and Nakamura, M. (1989) Biochem. Biophys. Res. Commun. **158**, 717–722

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

Iwasaki, M., Darden, T. A., Pedersen, L. G., Davis, D. G., Juvonen, R. O., Syeyoshi, T. and Negishi, M. (1993) J. Biol. Chem. **268**, 759–762

Jefcoate, F. (1978) Methods Enzymol. **52**, 255–279

Juvonen, R. O., Iwasaki, M. and Negishi, M. (1991) J. Biol. Chem. **266**, 16431–16435

Kronbach, T. and Johnson, E. J. (1991) J. Biol. Chem. **266**, 6215–6220

Lange, R., Maurray, L., Larroque, C. and Bienvenue, A. B. (1988) Eur. J. Biochem. **172**, 6465–6471

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

Lindberg, R., Burkhart, B., Ichikawa, T. and Negishi, M. (1989) J. Biol. Chem. **264**, 6465–6471

Lindberg, R. L. P. and Negishi, M. (1991) in Drug Metabolizing Enzymes: Genetics, Regulation and Toxicology (Proceedings of the Eighth International Symposium on the Microsomes and Drug Oxidations (Ingelman-Sundberg, M., Gustafsson, J.-A. and Orrenius, S., eds.), p. 135, Karolinska Institute, Stockholm

Matsunaga, T., Nagata, I., Holsztynska, E. J., Lapenson, D. P., Smith, A., Kato, R., Gelboin, H. V., Waxman, D. J. and Gonzalez, F. J. (1988) J. Biol. Chem. **263**, 17995–18002

Matsunaga, E., Zeugin, T., Zanger, V. M., Aoyama, T., Meyer, V. A. and Gonzalez, F. F. (1990) J. Biol. Chem. **265**, 17197–17201

Nebert, D. W., Nelson, D. R., Coon, M. J., Estabrook, R. W., Feyeresen, R., Fujii-Kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. J., Loper, J. C., Sato, R., Waterman, M. R. and Waxman, D. J. (1991) DNA Cell Biol **10**, 1–14

Negishi, M., Lindberg, R., Burkhart, B., Ichikawa, T., Honkakoski, P. and Lang, M. (1989) Biochemistry **28**, 4169–4172

Negishi, M., Yoshioka, H. and Aida, K. (1992) in Cytochrome P450 (Omura, T., Fujii-Kuriyama, Y. and Ishimura, Y., eds.), Kodansha, Tokyo, in the press

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

Oeda, K., Sakaki, T. and Ohkawa, H. (1985) DNA **4**, 203–210

Omura, T. and Sato, R. (1964) J. Biol. Chem. **239**, 2370–2378

Poulos, T. L. (1991) Methods Enzymol. **206**, 11–30

Sanger, F., Coulson, A. R., Barrel, B. G., Smith, A. J. H. and Roe, B. A. (1980) J. Mol. Biol. **143**, 161–178

Squires, E. J. and Negishi, M. (1988) J. Biol. Chem. **263**, 4166–4171

Tabor, S. and Richardson, C. C. (1987) Proc. Natl. Acad. Sci. U.S.A. **77**, 5201–5205

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