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ALTERATION OF THE SUBSTRATE SPECIFICITY OF MOUSE 2A *P*450s BY THE IDENTITY OF RESIDUE-209: STEROID-BINDING SITE AND ORIENTATION

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Summary—Mouse steroid 7α- and 15α-hydroxylases (*P*450c7 and *P*450c15) and coumarin 7-hydroxylase (*P*450coh) are structurally similar. To study the structural basis of the substrate specificities of these enzymes, we constructed a series of the mutant *P*450s, expressed in COS-1 and yeast cells, and studied them spectroscopically as well as enzyme-kinetically. A single amino acid mutation of residue-209 is sufficient to alter the substrate specificity of the *P*450s from xenobiotics to steroids and subsequently, from testosterone to corticosterone. Moreover, residue-209, when it is asparagine, appears to bind directly to the 11β-hydroxyl of corticosterone. The mutations also alter the spin equilibrium of *P*450 depending on the hydrophobicity and size of residue-209. We conclude, therefore, that residue-209 resides close to the 6th ligand of heme in the mouse 2A subfamily and is located at a critical site of the substrate-binding pocket. As a result, the identity of the residue-209 plays a key role in determining the substrate specificity.

INTRODUCTION

Collectively, mammalian *P*450s display a broad range of substrate specificities. Individually, however, they are characterized by a high degree of substrate and product selectivity. To understand the structural basis for the paradoxical characteristics of *P*450, it is essential to identify and modify its substrate-binding site in *P*450. This becomes a feasible objective and a major interest in *P*450 research, because of the recent advances in site-directed mutagenesis and heterologous expression systems [1, 2].

The mouse 2A subfamily (steroid 7α- and 15α-hydroxylases and coumarin 7-hydroxylase) provide an excellent model to study this structure–activity relationship since the subfamily members share high amino acid sequence similarities, yet exhibit different hydroxylase activities [2–5]. Therefore, we constructed a series of mutant *P*450s, expressed in COS-1 and yeast cells, and then investigated their spectral as well as enzymatic properties. In this article we summarize the current knowledge of the structural basis for steroid-substrate specificity of the mouse 2A subfamily.

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EXPERIMENTAL PROCEDURES

Construction and expression of mutant P450s

The details of the construction and expression were described in our previous publications [2–5]. Briefly, the cDNAs encoding wild-type *P*450c7, *P*450c15 and *P*450coh were inserted into M13 vector for the oligonucleotide-directed *in vitro* mutagenesis (Amersham) or pSELECT TM-1 vectors (Promega). Then, the mutated cDNAs were cloned in vectors pcD [2] and pAAH5 [6] for the expression in COS-1 and *Saccharomyces cerevisiae* AH22 cells, respectively.

Purification and analysis of mutant P450s

The mutant *P*450s were purified from the recombinant yeast microsomes using an aminooctyl Sepharose 4B and occasionally an additional hydroxylapatite column, then used for the spectral as well as enzyme-kinetic studies [4, 5]. Steroid hydroxylase and coumarin 7-hydroxylase activities were measured as described previously [7, 8].

RESULTS AND DISCUSSION

Mouse 2A subfamily

To date, three cytochromes *P*450 have been characterized for the mouse 2A subfamily:

*P*450c7, *P*450c15 (2A4) and *P*450coh (2A5) [9–11]. These *P*450s specifically catalyze steroid 7α- and 15α-hydroxylase and coumarin 7-hydroxylase activities, respectively. In spite of their divergent catalytic activities, the structures of these *P*450s are very similar. *P*450c15 and *P*450coh, for example, are 98% similar in structure, differing only by 11 amino acids within their 494 residues. *P*450c7, on the other hand, consists of 492 amino acids and shares 71% sequence similarity with *P*450c15.

The *P*450c15 gene is constitutively transcribed in the livers of female mice, while the transcription is repressed by growth hormone in the livers of male mice. As a result, *P*450c15 becomes female-specific in its expression in inbred mice [12]. *P*450c7 and *P*450coh, however, do not exhibit sex specificity in their expression. The mouse 2A *P*450s, therefore, differ not only in their catalytic functions but also in their gene regulations (Fig. 1).

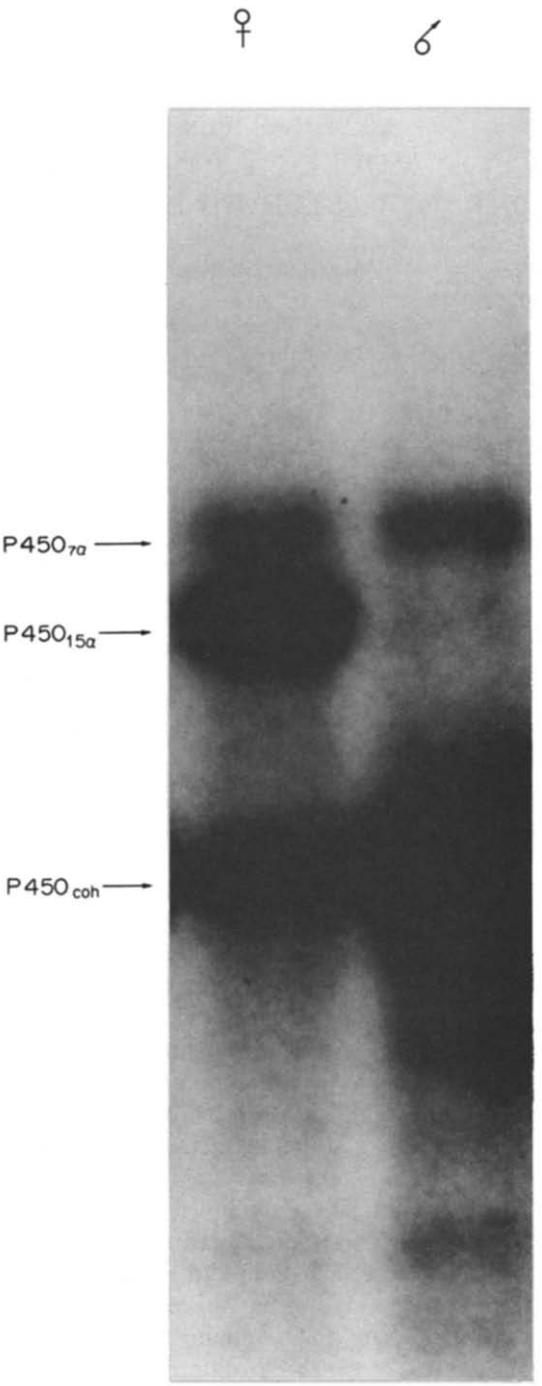

Fig. 1. The differential expressions of the mouse 2A subfamily. Total liver mRNAs were prepared from DBA/2J male and female mice and subjected to the processed Northern hybridization [12]. For this experiment, the double-stranded cDNAs were digested with *Cla* 1 and *Pst* 1, electrophoresed on an agarose gel and transferred to Nytran paper.

Alteration of substrate specificity from coumarin to steroids

The importance of residue-209 in determining the *P*450's substrate specificity, was discovered by expressing in COS-1 cells a series of mutants of *P*450coh in which each of the 11 different amino acids was substituted by the corresponding amino acids in *P*450c15 [3]. The amino acids at positions 117, 209 and 365 (Fig. 2) were identified as the critical residues for the hydroxylase activities. Residue-209 is leucine and phenylalanine in *P*450c15 and *P*450coh, respectively. It plays the most important role in determining the steroid-substrate specificity of the *P*450s, because a single amino acid mutation of Phe-209 to leucine is sufficient to alter the substrate specificity of *P*450coh from coumarin to steroids. Conversely, a mutation of Leu-209 to phenylalanine decreases the testosterone 15α-hydroxylase activity of *P*450c15 100-fold compared to wild-type *P*450coh. In addition, this mutant *P*450c15 acquires coumarin 7-hydroxylase activity, although the simultaneous mutations of residues -117, -209 and -365 are required to fully recover the activity level of wild-type *P*450coh [2, 3].

These results indicate, therefore, that the residues -117, -209 and -365 are located in the substrate-binding pocket. In particular, the identity of residue-209 is most critical for determining the specificity of steroid binding to the mouse 2A subfamily.

Alteration of substrate specificity from testosterone to corticosterone

Our previous study showed that wild-type *P*450c15 specifically catalyzes 15α-hydroxylation of $\Delta^4$3-ketone steroids including testosterone,

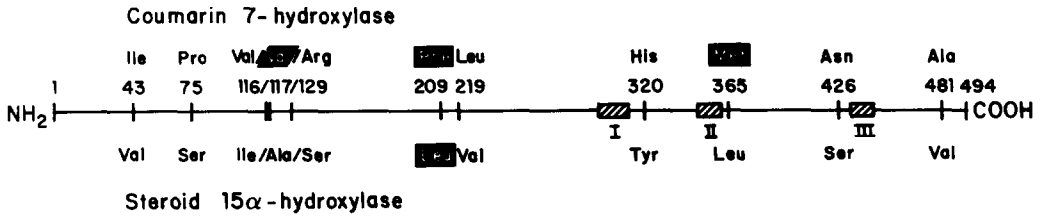

Fig. 2. Map of the 11 different amino acids between *P*450coh and *P*450c15 and the three residues responsible for their hydroxylase activities. The three residues important in determining the substrate specificities of the *P*450s are indicated by the white letters on black background. The hatched boxes with numbers I, II and III show the locations of the conserved motifs: the distal helix, the tridecapeptide, and a peptide containing the Cys residue which is the 5th ligand of heme.

progesterone and androstenedione, while the *P*450 exhibited little activity toward corticosterone [7–9]. The 11β-hydroxyl is the unique structural difference that separates corticosterone from the other 3-ketone steroids. We examined, therefore, the possibility that the 11β-hydroxyl is responsible for the steroid-substrate specificity of *P*450c15 [13].

The differential inhibitions of testosterone hydroxylase activities in *P*450c7 and *P*450c15 by corticosterone and deoxycorticosterone, first suggested the importance of the 11β-hydroxyl for corticosterone binding. Wild-type *P*450c7 contains asparagine at the position corresponding to Leu-209 in wild-type *P*450c15, and its activity was inhibited by corticosterone effectively. Corticosterone, on the other hand, did not inhibit the activity of wild-type *P*450c15. The mutant *P*450c7 in which Asn-209 was substituted by leucine, however, was no longer inhibited by corticosterone, whereas the mutant *P*450c15 having asparagine at position 209 became very sensitive to the steroid inhibition. This differential inhibition, therefore, depended on asparagine at position 209. Consistent with the importance of Asn-209 and 11β-hydroxyl for the steroid-binding specificity, deoxycorticosterone similarly inhibited the activity of the *P*450s regardless of the type of residue-209.

Furthermore, the significance of Asn-209 was shown more directly by measuring corticosterone-metabolizing activity of the wild-type and mutant *P*450c15. The mutant *P*450c15, in which asparagine substituted leucine at position 209, catalyzed the activity at 20 nmol/min/nmol *P*450. This activity level of the mutant *P*450coh was more than 20-fold higher than the wild-type *P*450coh. Reciprocally, the mutant *P*450c15 decreased its testosterone hydroxylase activity 10-fold; the activities of wild-type and mutant *P*450coh were 61.1 and 6.4 nmol/min/nmol *P*450, respectively [4]. This implies that the hydrogen bond may be formed between Asn-209 and 11β-hydroxyl and determines the efficient corticosterone binding to *P*450. Further, the mutant *P*450coh containing Asn-209 also catalyzed the high corticosterone-metabolizing activity at the same level as the mutant *P*450c15. Moreover, cortisol (an 11β-hydroxysteroid) was also metabolized by the mutants but not by the wild-type *P*450c15 and *P*450coh. The structures of metabolites formed from these 11β-hydroxysteroids have not been determined, however, several lines of evidence suggested that these metabolites were 15α-hydroxylated steroids.

The identity of residue-209 alters substrate specificity of the mouse *P*450s, as summarized in Table 1. We conclude, therefore, that the region containing residue-209 comprises a substrate-binding site which we designate as substrate-binding sequence 1 (SBS 1).

Topology of residue-209 in the mouse 2A subfamily

The crystal structure of bacterial *P*450cam, by aligning its amino acid sequence to the mammalian *P*450s, has been applied to predict the parts of mammalian *P*450 structure [14]. Numerous works have indicated that the heme-binding site and oxygen-binding pocket in bacterial and mammalian *P*450s are very well conserved both structurally and functionally [15–18]. The prediction, however, does not seem to apply in the case of residue-209 in the mouse 2A subfamily. According to the predicted mammalian *P*450 structure, residue-209 would be located far from the heme substrate pocket and substrate access channel [19]. However, our study on the spin alterations of

Table 1. The identity of residue-209 alters the substrate specificity of the mouse 2A subfamily

Residue-209	Preferred substrates
Phenylalanine	Coumarin
Leucine	Testosterone, Progesterone, Androstenedione
Asparagine	Corticosterone, Cortisol

the mutant *P*450coh and *P*450c15 suggested that residue-209 resides close to the 6th ligand of heme in the *P*450s [4]. The substitution of this residue with hydrophobic amino acids resulted in shifting the *P*450 to the high-spin state, while charged amino acids produced the low-spin *P*450. Moreover, *P*450 with a polar or a small hydrophobic amino acid exhibited a spectrum representing a mixture of the high- and low-spin forms (Fig. 3). Spin state is regulated by the hexacoordination of a water molecule to the 6th axial position of heme in *P*450; a *P*450 hexacoordinated to a water molecule is in the low-spin state, while the removal of this water molecule shifts the spin state of *P*450 from low to high [20]. Our findings, therefore, are dependent upon residue-209 residing close to the 6th ligand of heme in mouse *P*450s. A hydrophobic and/or large amino acid at position 209 creates an environment in which the heme can no longer be hexacoordinated to a water molecule, and therefore, converts the *P*450s to the high-spin forms. Recently, Gotoh has proposed a new sequence alignment of *P*4502A subfamily to the *P*450cam and identified six substrate recognition sites (SRS) in these mammalian *P*450s [21]. Unlike the previous Nelson and Strobel's alignment, the Gotoh's one maps residue-209 in SRS-2 which is located in F-G interhelical region.

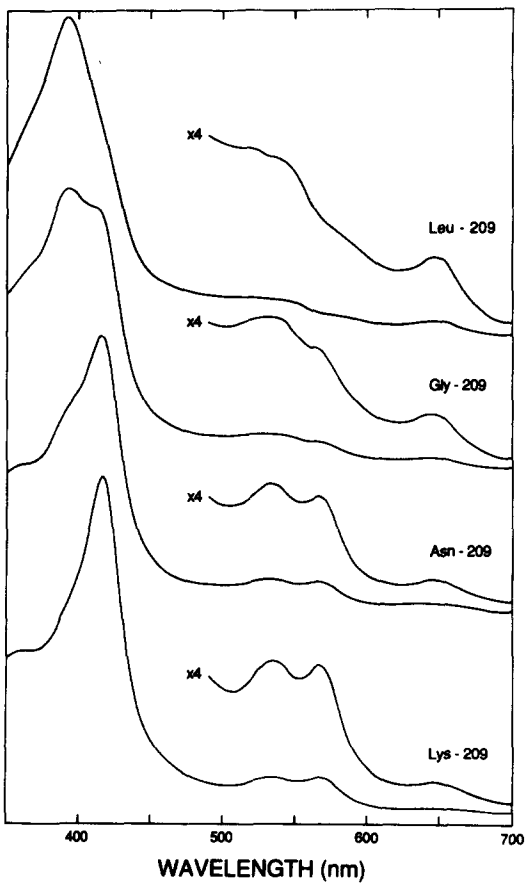

Fig. 3. Alteration of spin equilibrium of *P*450coh by mutation of residue-209. Each of mutant *P*450s was purified from recombinant yeast cells, dissolved in the buffer and used to measure the absolute-absorption spectrum [4]. The high-spin *P*450 displayed absorption peaks at 389 and 647 nm, while the peaks of low-spin spectrum appeared at 417, 534 and 570 nm.

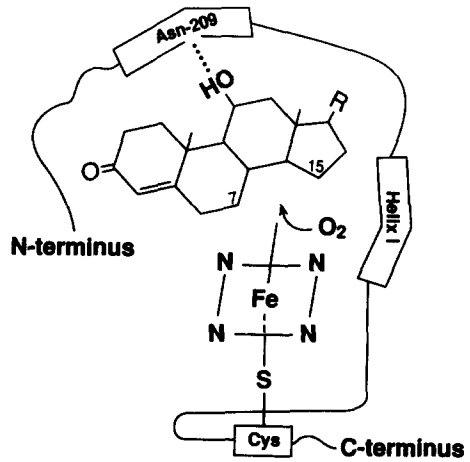

Fig. 4. Hypothetical structure of steroid-heme-binding pocket in the mouse 2A subfamily. This structure consists of protoheme, the fifth ligand (cysteine), helix I(oxygen-binding pocket), oxygen, 11β-hydroxysteroid and SBS 1 containing Asn-209.

CONCLUSION

The 6th axial position of heme is the active center of *P*450 where an oxygen molecule binds and is activated. In principle, therefore, the function of substrate-binding sites in *P*450 is to direct the reaction site of substrate molecule to the 6th axial position or its ligand. The 15α-position of a steroid, for example, should reside close to the 6th ligand in *P*450c15. Similarly, the steroid should direct its 7α-position to the ligand in *P*450c7. As a substrate-binding site, residue-209 apparently plays the key role in determining the orientation of the steroid molecule in the heme-substrate pocket of the mouse 2A subfamily. Based on the information obtained from our studies as well as by other researchers, we propose the hypothetical structure of heme-substrate pocket in the mouse *P*450s (Fig. 4), which emphasizes the role of residue-209 in the proper binding of the steroid. The structure does not, however, eliminate the importance of other amino acids for substrate binding in the *P*450s, since at least two or more

binding sites are necessary to fix a steroid molecule in the right orientation in the heme-substrate pocket. Other important amino acids include, for example, glutamine-302 in the oxygen-binding domain of aromatase *P*450, which is predicted to reside close to the C2-position of a substrate androstenedione [18]. In addition, arginine-346 in rat *P*450c17 is responsible for separating the 17α-hydroxylase activity from the lyase activity of the *P*450 [22]. Interestingly, this residue is located in the conserved sequence predicted as a steroid-binding region of *P*450 [23]. Furthermore, the high progesterone 21-hydroxylase activity of rabbit liver *P*4502C4 depends on the identity of residue-113 [24], while the simultaneous mutation of residues -58 and -114 alters the stereospecificity and regioselectively of testosterone hydroxylase activity in the rat *P*4502B1 [25]. A recent report shows that a single mutation of residue-478 also alters the regiospecificity of this rat *P*450 [26]. A future goal for our research is, therefore, to define the structural delineation of residue-209 in conjuction with these other amino acids in the substrate-binding pocket of the mouse 2A subfamily.

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