Reciprocal Size−Effect Relationship of the Key Residues in Determining Regio- and Stereospecificities of DHEA Hydroxylase Activity in P450 2a5

Tomohide Uno, Eddy Mitchell,[‡,§] Kaoru Aida, Mill H. Lambert,[||] Thomas A. Darden,[‡] Lee G. Pedersen,[‡] and Masahiko Negishi*

Pharmacogenetics section, Laboratory of Reproductive and Developmental Toxicology and Laboratory of Quantitative and Computational Biology, National Institute of Environmental Health Sciences, National Institutes of Health and Glaxo Wellcome Research and Development, Research Triangle Park, North Carolina 27709

Received October 23, 1996; Revised Manuscript Received December 11, 1996[⊗]

ABSTRACT: Collectively, the P450 2a4/2a5 system hyrdoxylates DHEA in at least three positions (7α, 7*β*, and 2α). An individual P450, however, exhibits high specificity to one of these products. Using site-directed mutagenesis of *m*P450 2a5 from the wild mouse *Mus minutoides* and bacterial expression, we have associated the function of residues 117, 209, and 481 with the respective specificity observed in each P450. Ala at position 117 determines the 7*β*-hydroxylase activity, whereas Val at this position defines the 2α-hydroxylase activity. Leu at position 209 is essential for high DHEA 7α-hydroxylase activity. The substitutions of residue 481 with various hydrophobic amino acids elicited a profound alteration of the specific hydroxylation rates, but did not influence the regio- and stereospecificities at either of the three positions of DHEA. The alterations caused by residue 481 also depended on the residue identity at position 117 or 209. The results indicate that the sizes of several key residues obey a concerted reciprocal relationship whereby the substrate pocket of the P450s adjusts to accommodate DHEA. A limited molecular modeling study successfully correlates DHEA binding to experimental DHEA hydroxylase activities for a series of mutants at key positions.

Mouse P450 2a5 is characterized as coumarin 7-hydroxylase (Negishi et al., 1989). Although P450 2a4 differs by only 11 amino acid residues from P450 2a5, it does not catalyze coumarin 7-hydroxylase activity but instead exhibits high Δ^4, 3-ketosteroid 15α-hydroxylase activity (Lindberg et al., 1989). Since the P450 2a4 gene is not present in the wild mouse strain *Mus spretus*, P450 2a5 appears to be ancestral to P450 2a4 (Aida et al., 1994). The substitution of Phe at position 209 to Leu in an ancestral P450 2a5 gene seems to have been the key event in the evolution of P450 2a4, as this single amino acid substitution efficiently converts the substrate specificity of P450 2a5 from coumarin to steroid hydroxylase activity (Lindberg & Negishi, 1989). Using site-directed mutagenesis, we identified several critical residues that determine the specificity of P450 2a4/2a5 activity. These include residues 117, 365, and 481 in addition to residue 209 (Negishi et al., 1996). Importantly, the residues at corresponding positions in other P450s (rat P450 2B1 and rabbit P450s 2C, for example) also play critical roles in altering their activities (Kedzie et al., 1991; Straub et al., 1983; Luo et al., 1994; Richardson & Johnson, 1994). As a result, the substrate specificity of these P450s can be determined by the residue identities at a few common positions such as 117, 209, 365, and 481 in the mouse P450s. When the amino acid sequences of the mammalian P450s are aligned with the sequences of bacterial P450s for which three-dimensional structures have been resolved (Gotoh, 1992; Hasemann et al., 1995), all of the key residues of the mammalian P450s are found to reside in the substrate−heme pocket. We also aligned these key residues of the mouse P450s to residues that contact the substrate in bacterial P450 101 (Iwasaki et al., 1995a).

Despite the fact that the substrate specificity of a given P450 can be altered by an amino acid mutation at a few key positions, the P450 enzymes exhibit, as an intrinsic characteristic, a broad range of substrate and/or product specificities. The molecular mechanism by which a P450 recognizes various structurally unrelated chemicals as substrates is not, however, well understood. To visualize the binding of substrate to these P450 enzymes, we performed site-directed mutagenesis studies in order to associate the key residues in the pocket of the P450 2a4/2a5 with specific sites of different steroids. For example, residue 117 may be near the 3*β*-hydroxy group of DHEA, since the substitution of this residue alters DHEA but not androstenedione hydroxylase activity (Iwasaki et al., 1994). The 11*β*-hydroxy group of corticosterone may be directed toward the residue-209 in the P450s, since placing Asn at this position creates a novel corticosterone hydroxylase activity (Iwasaki et al., 1993). These results were employed in order to model the binding orientations of steroid with respect to the location of key amino acid residues in the substrate−heme pocket (Iwasaki et al., 1994, 1995a,b). The models suggested that the P450 pocket may be structurally flexible so as to accommodate various substrates in multiple orientations.

Collectively, the P450 2a4/2a5 system hydoxylates DHEA with the appearance of at least three different products: 7αOH, 7*β*OH, and 2αOH DHEAs (Iwasaki et al., 1995b). We have shown previously that the residues 117 and 209 play important roles in providing the P450s with DHEA hydroxylase activity (Iwasaki et al., 1994; 1995a). For instance, the substitution of Phe-209 in P450 2a5 to Leu or

[‡] Laboratory of Quantitative and Computational Biology.
[§] Supported by NIEHS Training Grant, ES-07266.
[||] Glaxo Wellcome Research and Development.
[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1997.

S0006-2960(96)02654-2 CCC: $14.00

Val alters the relative regiospecificity of DHEA 2- and 7-hydroxylase activities (Iwasaki et al., 1995b). However, the roles of individual residues at positions 117, 209, and 481 in determining the regio- and stereospecificities of the DHEA 7α-, 7β-, and 2α-DHEA hydroxylase activities have not been clarified. As an extension of our study of the evolution of the P450 2a4/2a5 system (Aida et al., 1994), we have cloned and bacterially expressed *m*P450 2a5 cDNA from the wild mouse *Mus minutoides*. We have, somewhat fortuitously, discovered that the regio- and stereospecificities of *m*P450 2a5 differ from those of either *d*P450 2a5 or P450 2a4 of the domestic inbred mice. Subsequently, we have substituted the residues at positions 117, 209, and 481 in *m*P450 2a5 with various hydrophobic amino acids and measured their DHEA hydroxylase activities. This paper will discuss how the side-chain size of these specific residues may play a defining role in the determination of the P450 substrate and product specificities.

EXPERIMENTAL PROCEDURES

cDNA Cloning of mP450 2a5. RNAs were prepared from *Mus minutoides* livers (kindly provided by Dr. M. Potter, National Cancer Institute, NIH, Bethesda, MD) using guanidine isothiocyanate extraction and CsCl centrifugation. mRNAs were primed with oligo dT, transcribed with M-MLV reverse transcriptase Superscript (BRL), then incubated with *E. coli* DNA polymerase, RNase H, and *E. coli* DNA ligase (BRL) to synthesize the double-stranded cDNAs. The double stranded cDNAs were ligated to *Eco*RI-*Not*I adaptor (Pharmacia) and then to *Eco*RI site of ZAP II vectors. The resulting liver cDNA library was screened by the *Cla*I-*Cla*I fragment of *d*P450 2a5 cDNA. The obtained clones were subcloned to M13 phage vectors and sequenced.

Construction of Mutant P450s. Site-directed mutagenesis was performed by using the Transformer Site-directed mutagenesis kit (Clontech, Palo Alto, CA). The following oligonucleotides were used: 5′-AAAGACTATGGCGTAGTCTTCAGCAGCGGGGAAC-3′ and 5′-AAAGACTATGGCGTACTCTTCAGCAGCGGGGAAC-3′ to mutate Ala at position 117 to Val and Leu, respectively; 5′-ATGATGTTGGGAAGCCTCCAGTTCACCGCTACC-3′ and 5′-ATGATGTTGGGAAGCAACCAGTTCACCGCTACC-3′ to substitute Phe at position 209 to Leu and Asn, respectively; 5′-CCTAGACTTGTGGGCTTTGGTACGATCCCACCAAAC-3′, 5′-CCTAGACTTGTGGGCTTTGTCACGATCCCACCAAAC-3′, and 5′-CCTAGACTTGTGGGCTTTCTCACGATCCCACCAAAC-3′ to change Ala at position 481 to Gly, Val, and Leu, respectively; and 5′-CAGAGATTTGCAGACATGATCCCCATGGGCC-3′ and 5′-CAGAGATTTGCAGACCTGATCCCCATGGGCC-3′ to mutate residue-365 to Met and Leu, respectively. The mutated codons are underlined. Using these mutated P450s, various chimeric proteins were constructed for the mutant P450s containing different combinations of substitutions at positions 117, 209, and 481. For this, a P450 cDNA was cloned into *Nde*I (5′) and *Xba*I (3′) sites of pUC19 and digested with the internal *Stu*I (707 bp downsteam from the initiation site) and *Xba*I to generate the 1.0 kbp *Stu*I−*Xba*I and 3.4 kbp *Xba*I−*Stu*I fragments. These two DNA fragments from the mutant P450s were religated in various combinations. The cDNAs were sequenced to confirm the mutations and designated by the amino acid codes for positions 117, 209, and 481 (the wild type *m*P450 2a5, for example, is represented by AFA).

Bacterial Expression and Purification of P450. The recombinant *E. coli* DH5α cells transformed with the P450 cDNAs were cultured under the conditions previously described (Sueyoshi et al., 1995). Harvested bacterial cells were resuspended in 20 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.2 mM EDTA, 0.1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL of leupeptin. After being sonicated, the cell homogenate was centrifuged at 38 000 rpm for 1 h in a 45Ti rotor and the pellets were washed with the buffer. The resulting bacterial membranes were solubilized in 100 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.2 mM EDTA, 0.1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL of leupeptin and 0.2% Emulgen 911 (gift from KaoAtlas, Japan) and was centrifuged at 38 000 rpm for 1 h. The solubilized P450 was purified by Octyl-Sepharose CL-4B and hydroxyapatite columns as previously described (Sueyoshi et al., 1995). A typical purified fraction contained 13 nmol of P450/mg of protein. We observed a large difference in recovery of the purified proteins depending on the type of the mutants P450. The recoveries varied from approximately 10−20 nmol of the wild-type P450 to 1−0.5 nmol of some mutant P450s from 10 L of bacterial culture.

Computed Binding Energy. Using the binding orientations modeled previously (Iwasaki et al., 1995b; Negishi, et al., 1996) as basis for the initial docking coordinates, the Molecular Viewing Program MVP program was employed to search for optimal DHEA binding positions to modified P450cam. MVP is a multipurpose program for the analysis and prediction of molecular structure employed extensively at Glaxo Wellcome. With the application of MVP, we were able to avoid the possible bias of manual docking methods and to include the effect of solvent. This general purpose program calculates binding energy, based on the DISCOVER CFF 91 force field (Maple et al., 1994), with optimization of side-chain orientation; using a combination of a buildup procedure (Vasquez & Scheraga, 1985), Monte Carlo moves the position and orientation and energy minimization of the pocket and ligand degrees of freedom. Solvation effects are modeled by an electrostatic term together with hydrophobic surface tension terms (Ooi et al., 1987; Lambert, 1996).

Analytical Methods. P450 and protein contents were determined according to the methods of Omura and Sato (1964) and Bradford (1976), respectively. Reconstitution mixtures (0.5 mL) for the P450 activities contained 100 mM potassium phosphate buffer, pH 7.5, purified P450 (unless specified, 50 pmol), rat NADPH-P450 reductase (250 pmol), $MgCl_2$ (5 mM), dilauroylphosphatidylcholine (25 μg), NADPH (5 mM) and substrates, DHEA (50 μM), testosterone (100 μM), or coumarin (25 μM). Testosterone 15α-hydroxylase, DHEA hydroxylase, and coumarin 7-hydroxylase activities were measured by the methods described previously (Harada & Negishi, 1989; Iwasaki et al., 1994; Kaipainen et al., 1985).

RESULTS AND DISCUSSION

mP450 2a5 Cloned from the Wild Mouse Mus minutoides. Two different cDNAs were cloned for P450 2a5 from the livers of *Mus minutoides*. Although the exonic sequence was identical in both the P450 cDNAs, there was a single

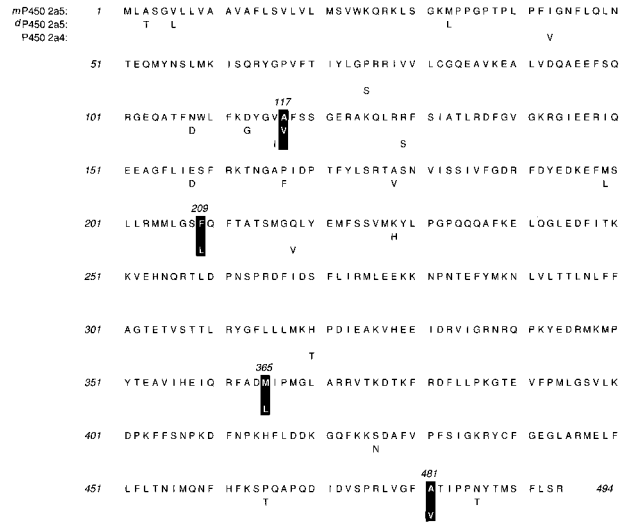

FIGURE 1: Amino acid sequence of *m*P450 2a5 and comparison with *d*P450 2a5 and P450 2a4. The full amino acid sequence of *m*P450 2a5 is shown, while the amino acid residues that differed from *m*P450 2a5 are indicated for *d*P450 2a5 and P450 2a4. The four key residues are boxed with the number of their positions on top.

nucleotide difference in their 3′-noncoding regions. Using the *Dde*I site polymorphism created by this nucleotide mutation, we measured the numbers of the two P450 cDNAs. Approximately half of 38 clones analyzed were digested by *Dde*I. Thus, the two cDNAs may define different alleles. However, the possibility that the cDNAs may be encoded by duplicated genes in *Mus minutoides* cannot be ruled out at the present time. Consistent with previous finding that the P450 2a4 gene is not present in the other wild mouse *Mus spretus* (Aida & Negishi, 1994), no P450 2a4 cDNA was recovered from *Mus minutoides*. We designated the P450 2a5 of *Mus minutoides m*P450 2a5 and the corresponding P450 in domestic mouse *d*P450 2a5. The amino acid sequence of *m*P450 2a5 differed by 13 and 9 residues from those of *d*P450 2a5 and P450 2a4, respectively (Figure 1). The identities of the key residues for *m*P450 2a5 are Ala, Phe, and Ala at positions 117, 209, and 481, respectively. The corresponding residues in *d*P450 2a5 are Val, Phe, and Ala and Ala, Leu, and Val in P450 2a4.

Substrate Specificity of mP450 2a5. Since we previously showed that placing Ala at position 117 results in a drastic decrease of coumarin 7-hydroxylase activity in *d*P450 2a5 from *Mus musculus domesticus* (Lindberg & Negishi, 1989), we were not surprised to find that *m*P450 2a5 exhibited more than a 10-fold reduced coumarin 7-hydroxylase activity compared with *d*P450 2a5 (Table 1). Similarly, because of the Phe residue at position 209, testosterone 15α-hydroxylase activity of *m*P450 2a5 was found to be 50-fold lower than that of P450 2a4. Thus, *m*P450 2a5 is characterized as an enzyme having low coumarin and testosterone hydroxylase activities. When DHEA was used as substrate, *m*P450 2a5 yielded *only* 7*β*OH DHEA, whereas *d*P450 2a5 produced *only* 2αOH DHEA (Table 1). P450 2a4, on the other hand, metabolized DHEA to 7αOH DHEA as the major product and 7*β*OH DHEA and 2αOH DHEA as the two minor products. As a result, these P450s exhibited high regio- and stereospecific DHEA hydroxylase activity, although the specific activities were lower than the testosterone 15α-hydroxylase activities in P450 2a4 (40 nmol/min/nmol of P450).

Table 1: Substrate and Product Specificities of the Wild-Type P450s. Hydroxylase Activity of the P450s Was Reconstituted as Described in the Experimental Procedures. *m*P450 2a5, *d*P450 2a5, and P450 2a4 Are Designated by *m*2a5, *d*2a5, and 2a4, Respectively. Substrates Used Were Dehydroepinadrosterone (DHEA), Testosterone (T), and Coumarin (COH). Products Formed Were 7*β*-Hydroxy DHEA (7*β*OH), 7α-Hydroxy DHEA (7αOH), 2α-Hydroxy DHEA (2αOH), 15α-Hydroxy Testosterone (15αOH) and 7-Hydroxy Coumarin (7OH)

	DHEA				
	7*β*OH[a]	7αOH	2αOH	T 15αOH	COH 7OH
*m*2a5	0.4 ± 0.0	nd	nd	0.8 ± 0.0	0.5 ± 0.0
*d*2a5	nd	nd	1.3 ± 0.0	0.5 ± 0.0	7.5 ± 0.2
2a4	0.4 ± 0.0	1.5 ± 0.0	0.2 ± 0.0	41 ± 1.6	nd

[a] nmol of product formed/min/nmol of P450. [b] nd = activity not detectable.

Table 2: Substrate and Product Specificities of the Mutant *m*P450s 2a5. Mutant P450s Are Indicated by the Amino Acid Identities at Positions 117, 209, and 481 Using Single-Letter Amino Acid Designations. Mutant AFG, for Example, Contains Ala, Phe, and Gly at These Positions, Respectively. The Wild-Type *m*P450 2a5 (AFA) Is Designated by an Asterisk. All of the Mutant P450s with Phe or Leu at Position 209 Are Summarized Separately in A and B, Respectively

	DHEA				
	7*β*OH[a]	7αOH	2αOH	T 15αOH	COH 7OH
			A		
AFG	1.2 ± 0.1	nd	nd[b]	4.9 ± 0.0	0.5 ± 0.0
AFA*	0.4 ± 0.0	nd	nd	0.8 ± 0.0	0.5 ± 0.0
AFV	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	01 ± 0.0	0.6 ± 0.0
VFG	nd	nd	0.8 ± 0.0	2.2 ± 0.0	0.3 ± 0.0
VFA	nd	nd	0.4 ± 0.0	0.8 ± 0.0	2.9 ± 0.0
VFV	nd	nd	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.0
VFL	nd	nd	0.4 ± 0.1	0.1 ± 0.0	nd
LFG	nd	nd	nd	nd	0.1 ± 0.0
LFA	nd	nd	nd	nd	0.1 ± 0.0
			B		
ALG	1.8 ± 0.2	1.2 ± 0.2	nd	26 ± 1.3	0.2 ± 0.0
ALA	11 ± 0.1	3.4 ± 0.0	nd	17 ± 0.5	0.1 ± 0.0
ALV	0.4 ± 0.0	1.6 ± 0.1	nd	16 ± 0.1	0.1 ± 0.0
ALL	0.0 ± 0.00	0.1 ± 0.0	nd	nd	nd
VLG	nd	nd	nd	2.1 ± 0.3	nd
VLA	nd	0.8 ± 0.0	2.6 ± 0.1	4.4 ± 0.1	0.9 ± 0.0
VLV	nd	0.3 ± 0.1	0.2 ± 0.0	1.2 ± 0.3	0.7 ± 0.0
VLL	nd	0.7 ± 0.1	0.5 ± 0.1	4.3 ± 0.0	nd

[a] nmol of product formed/min/nmol of P450. [b] nd = activity not detectable.

Residue 117 Defines DHEA 7β- or 2α-Hydroxylase Activity. There are 13 residue differences between *m*P450 and *d*P450 2a-5. Among these, only position 117 has a different amino acid at a key position (117, 209, 365, or 481). Thus, we examined the Ala-117 substitution to discern if it is responsible for DHEA 7*β*-hydroxylase activity in *m*P450 2a-5. The substitution of Ala-117 with Val (mutant VFA) converted the specificity of *m*P450 from DHEA 7*β*- to 2α-hydroxylation (Table 2). Conversely, the Val to Ala substitution altered the *d*P450 2a-5 specificity from DHEA 2α- to 7*β*-hydroxylase activity (data not shown). The results clearly indicate that the residue identity at position 117 is the key factor in determining whether P450 2a5 is a DHEA 7*β*- or 2α-hydroxylase: Ala and Val at this position determines 7*β*- and 2α-DHEA hydroxylase activities, respectively. Although the mutants AFV and AFL have Ala at position 117, they produced 7*β*OH DHEA in addition to

Table 3: Substrate and Product Specificities of P450s 2a5 with Mutation of Residue-365. The Arrows Indicate the Amino Acid Mutations Which Are Represented by the Single-Letter Designations

	DHEA				
	7βOH[a]	7αOH	2αOH	T 15αOH	COH 7OH
*m*2a5 M → L	0.3 ± 0.0	nd[b]	nd	0.2 ± 0.00	nd
*d*2a5 M → L	nd	nd	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.0
2a4 L → M	0.2 ± 0.0	0.3 ± 0.0	nd	7.5 ± 0.3	nd

[a] nmol of product formed/min/nmol of P450. [b] nd = activity not detectable.

2αOH DHEA. These activities, including the third product, 7αOH DHEA, were very low however, and the two mutant P450s lost their regio- and sterospecificties. Thus, the production of 2αOH DHEA by the mutant P450s could not be considered as a specific alteration by Ala at position 117. The placement of Leu at position 117 of *m*P450 2a5 abolished DHEA as well as testosterone hydroxylase activities, although the mutant P450s retained detectable levels of coumarin 7-hydroxylase activity.

Residue 209 Defines DHEA 7α-hydroxylase Activity. The only wild-type P450 that catalyzed DHEA 7α-hydroxylase activity was P450 2a4 (Table 1). Among the three key residues (positions 117, 209, and 481) that determine the P450 activity, *m*P450 2a5 differs in the residues at the latter two positions from P450 2a4: Phe and Leu at position 209, respectively, and Val and Ala at position 481, respectively (Figure 1). First, we examined whether residue 209 plays a role in specifying DHEA 7α-hydroxylase activity of the P450s. For this, Phe at position 209 of *m*P450 2a5 was substituted with Leu, expressed in bacterial cells and purified. As expected from our original finding (Lindberg & Negishi, 1989), this mutant *m*P450 2a5 with Leu at position 209 increased testosterone 15α-hydroxylase activity dramatically and decreased further an already low coumarin 7-hydroxylase activity (mutant designated ALA in part B of Table 2). Most importantly, however, the mutant was found to catalyze the 7α-hydroxylase activity at a high level (3.4 nmol/min/nmol). In addition to the 7α-hydroxylase activity, the Leu-209 mutant *m*P450 2a5 also catalyzed DHEA 7β- or 2α-hydroxylase activity depending on whether position 117 was Ala or Val. Consistent with the fact that all Phe/209-mutants (Phe at position 209) did not catalyze DHEA 2α-hydroxylase activity, none of the Leu/209-mutants with Ala-117 exhibited 2α-hydroxylase activities. Conversely, the Leu/209-mutants with Val-117 showed no 7β-hydroxylase activity (Table 2). Nevertheless, the smaller (than Phe) amino acid (such as Leu) at position 209 appeared to be essential for providing the P450s with high DHEA 7α-hydroxylase activity. This confirms our previous findings that the DHEA 7α-hydroxylase activity of *d*P450 2a5 is increased by the substitution of Leu-209 with an smaller amino acid Val (Iwasaki et al., 1995).

Residue 365 Does Not Alter the Specificities. We mutated Met-365 of the P450s 2a5 to the corresponding Leu in P450 2a4 and Leu-365 of P450 2a4 to Met. These P450s with mutation of residue 365 were expressed in bacterial cells, purified, and used to measure the hydroxylase activities (Table 3). These 365-mutant P450s exhibited the lower specific activities of the DHEA, testosterone, and coumarin hydroxylations compared with activities of the corresponding wild-type P450s. The mutant P450s, however, retained the same regio- and stereospecificities as the corresponding wild-type P450s: in the case of the DHEA hydroxylase activity, mutant *m*P4502a5 produced 7βOH DHEA, mutant *d*P4502a5 2αOH DHEA and P4502a4 7αOH DHEA.

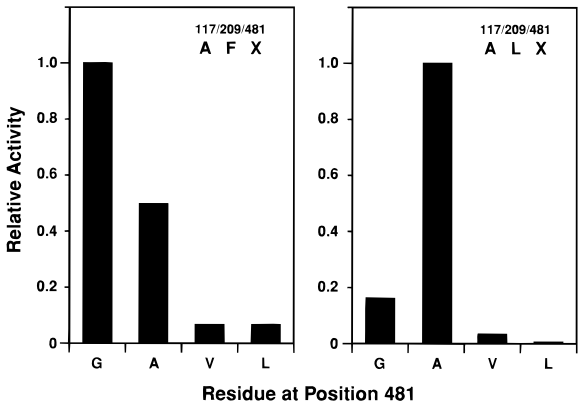

FIGURE 2: Graphic representation of reciprocal size−effect relationship of residues 209 and 481 on DHEA 7β-hydroxylase activity. The relative activities were calculated by taking the highest activity in each group to be 1.0. Single-letter designations are used to show amino acid residues. The letter X for each mutant indicates the various residues at position 481. These are described at the bottom of each panel.

Residue 481 Alters DHEA Hydroxylation Rates. We constructed and expressed a series of mutant P450s by substituting residue 481 with Gly, Ala, Val, or Leu. From these studies we found that the size of the side-chain at residue 481 elicited a profound effect on the DHEA hydroxylation rates in the P450s. When the smallest residue Gly was placed at position 481, the Phe/209-mutant P450s exhibited the highest DHEA 7β-hydroxylase activity. Moreover, the activity of the Phe/209-mutants decreased as the size of the hydrophobic amino acid at position 481 became larger (Table 2). Interestingly, when the smaller Leu replaced Phe at position 209 (Leu/209-mutants), the larger (than Gly) Ala at position 481 provided the highest DHEA 7β-hydroxylase activities (Table 2 and also visualized in Figure 2). Similarly, the rate of DHEA 2α-hydroxylase activity was also altered by the residue at position 481, depending on the residue at position 209 in the mutant P450s. Again, Gly-481 provided Phe/209-mutant P450 with the highest 2α-hydroxylase activity, whereas Ala-481 conferred the highest activity to the Leu/209-mutant P450s (Table 2). The hydroxylation rates were decreased by increasing the size of residue 481 regardless of whether position 209 was occupied by Phe or Leu. However, the P450s with Leu-481 exhibited higher DHEA 2α-hydroxylase activity than the P450s with Val-481. To a lesser degree, the DHEA 7α-hydroxylation rate was altered depending on the size of residue 481. Despite the fact that the side-chain size of residue 481 profoundly affected the hydroxylation rates, the regio- and stereospecificities at either the 7β-, 7α-, or 2α positions, which were predetermined by the identities of the residues at positions 117 and 209, were not altered.

Alteration by Residue 481 of Testosterone and Coumarin Hydroxylase Activities. Intriguingly, the mutant *m*P450 2a5 that has Gly at position 481 acquired a relatively high testosterone 15α-hydroxylase activity, although the residue at position 209 remained Phe in this P450 (Table 2). The activity of this mutant P450 was approximately 6-fold higher than that of the wild-type *m*P450 2a5. This activity was decreased as the size of residue 481 became larger. The same size-dependent change at residue 481 for testosterone

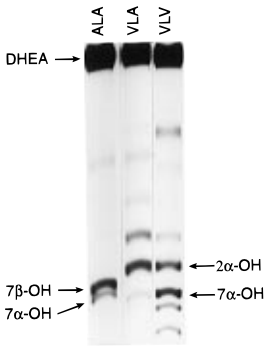

FIGURE 3: Thin layer chromatography of DHEA metabolites formed by mutant mP450 2a5. Since the specific hydroxylase activities varied, different reaction conditions were used for each mutant P450 in order to obtain similar intensities of the radioactive DHEA metabolites. Mutant ALA, 40 pmol of this enzyme was incubated for 30 min; mutant VLA, 100 pmol for 1 h; mutant VLV, 200 pmol for 1 h. The reaction mixtures were dried, chromatographed on silica gel, and exposed to X-ray film.

15α-hydroxylase activity was observed with mutant P450s regardless of whether Ala or Val was at position 117. When Leu was placed at position 209, the 15α-hydroxylase activity did not depend on the residue sizes at position 481. With respect to the evolution of testosterone 15α-hydroxylase activity (P450 2a4 enzyme), it is reasonable to speculate that the appearance of Gly at position 481 might have been the substitution that originally conferred this activity to an ancestral P450 2a5. Nature appears to have selected the Phe to Leu substitution at position 209 rather than the Gly substitution at position 481, since the 209 substitution provides the P450 with higher activity toward testosterone.

The wild-type *m*P450 2a5 exhibited a low coumarin 7-hydroxylase activity (Table 1). In general, the substitutions of residue 481 do not affect already low coumarin hydroxylase activity of the P450s. The exception is the mutant VFA which exhibits a relatively high coumarin 7-hydroxylase activity (5-fold higher than that of the wild-type *m*P450 2a5 (Table 2)). Moreover, the substitution of residue 481 with either a smaller Gly or a larger Val decreased the activity of the P450s. The effect of the alteration at residue 481 also depended on the larger residues at positions 117 and 209, since the effect was not unambiguously seen in the P450s with the smaller Ala (than Val) and Leu (than Phe) at these positions, respectively (Table 2). The results indicate that a high level of coumarin 7-hydroxylase activity is accomplished by the proper combination of residue sizes at positions 117 and 481.

Broadening the Regio- and Stereospecificities as the Specific Hydroxylation Rate Decreases. When the artificial mutations of the amino acid residues were introduced, the regio- and stereospecificites of the mutant P450s were additionally broadened, as the specific activity of the mutant P450s was decreased. This broadening was generally observed with the mutants having Ala and Phe at positions 117 and 209, respectively (Table 2). These mutant P450s exhibited the highest and most regio- and stereospecific DHEA hydroxylase activity when the residue at position 481 was Gly. The activity as well as the specificity were decreased as the size of residue 481 became larger. Paradoxically, however, some other mutant P450s broadened the general specificity while retaining a high specificity at certain positions of DHEA. Examples for this are shown in Figure 3. The DHEA hydroxylase activities (7αOH + 7*β*OH + 2αOH DHEAs) of these mutants ALA, VLA, and VLV were 14, 2, and 0.5 nmol/min/nmol of P450, respectively. As the specific activity (hydroxylation rate) of the P450s decreased, the number of DHEA products increased: mutant *m*P450 VLA produced six detectable metabolites including 2αOH and 7αOH DHEA, while mutant VLV generated at least eight products (Figure 3). In spite of these broadened specificities, these P450s with Val-117 did not hydroxylate the 7*β*-position of DHEA.

Table 4: Calculated Binding Energies for DHEA in Three Orientations in the Substrate Pocket of P450cam. The Energy Values (kcal/mol) Are Relative to That of DHEA in the 7*β*-Orientation in AFG, i.e., Erel,x = Ex − EAFG. For Mutants, Thr, Thr, and Val at Positions 101, 184, and 396, Respectively, in P450cam Were Substituted for the Residues 117, 209, and 481, Respectively, in P450 2a5 (Negishi et al., 1996)

	binding orientation		
	7*β*OH	7αOH	2αOH
AFG	0	10	66
AFA	25	32	142
VFA	35	32	143
VFV	64	71	147

DHEA-Binding Energies and Side-Chain Sizes. The computed energies of each DHEA binding orientation (7*β*-, 7α-, and 2α-) in various mutants P450 were calculated (Table 4). Since the MVP program did not allow for movement of the protein backbone, these energies were dominated by steric effects. As a result, the energy values are somewhat inflated compared with those obtained using AMBER which relieved steric clashes fully and moved the P450cam backbone on the order of 1 Å in binding DHEA. Given the limitation of the method, the present modeling provides a quantitative beginning for understanding trends in the regio- and stereospecificities of DHEA hydroxylase activity in the mutants P450. When Phe is placed at position 209, we compute that the binding energies (Table 4) in the 7*β*- orientation are progressively less favorable as the residue-481 becomes larger, in agreement with the changes of 7*β*-hydroxylase activities in these mutants (Table 2). Moreover, the placement of Ala at position 117 gives a more favorable (less) binding energy than that of Val, a result consistent with the fact that the 7*β*-hydroxylase activity requires Ala at position 117. To a lesser degree, a similar trend is also observed for computed DHEA binding energies (Table 4) in the 7α-orientation, although the corresponding activities are too low to detect. The calculated binding energies for the 2α-orientation, however, are always larger than those for the 7α- and 7*β*-orientations; thus, the energies do not predict the change from 7*β*- to 2α-hydroxylations by the substitution of Ala-117 to Val. This disagreement between energy and activity suggests that our current 2α-binding orientation requires further refining or that the P450cam structure is not a sufficiently precise model for our application.

General Discussion. P450 enzymes are capable of altering substrate and product specificities so as to create novel activities by single amino acid substitutions (Negishi et al., 1996). Since the substitutions are often those of Val to Ala, Ala to Gly, Leu to Ile, and Phe to Leu, the size of the side chains of the hydrophobic amino acids is implicated as a major factor for alteration of P450 activity (Negishi et al., 1996). In a previous report (Iwasaki et al., 1995a), we demonstrated that the regiospecificity of the P450s 2a4/2a5

at the 15α position of androstenedione is increased as the size of residue 481 becomes larger. Similar size−effects of substrate specificity have also been reported in the other P450s (Kedzie et al., 1991; Straub et al., 1983; Luo et al., 1994; Richardson & Johnson, 1994). Our present studies have provided further evidence that the size of the amino acid side chains, indeed, can determine the regio- and stereospecificites of steroid hydroxylase activity in the P450s. Moreover, the present studies have now suggested that the residues at several key positions define a reciprocal size−effect relationship in order to achieve the high rates of specific P450 activities. This effect is typically seen with the P450s for which Gly-481 provides P450s with Phe at position 209 the highest DHEA 7β-hydroxylase activity, whereas Ala-481 confers the highest activities to P450 with Leu at position 209. It thus appears that substitutions at key residues may lead to adjustment of the size and shape of the substrate pocket without changing a backbone structure so as to accommodate the hydroxylation of DHEA.

How a given residue's size affects P450 activity also depends on the sizes (or properties) of the substrates. In a series of P450s having Phe at position 209, testosterone is most efficiently hydroxylated by the P450s with Gly at position 481, whereas the smaller coumarin is hydroxylated at a higher rate when the larger Ala is placed at position 481 in the P450s. The correlation between the size of substrate and key residues, however, is less clear in some other mutant P450s (particularly in the P450s with Leu at position 481), suggesting that other factors such as the modification of backbone structure or side-chain orientation must be considered in order to fully understand the alterations caused by these substitutions.

REFERENCES

Aida, K., Moore, R., & Negishi, M. (1994) *Genomics 19*, 564−566.

Bradford, M. (1976) *Biochemistry 72*, 248−258.

Gotoh, O. (1992) *J. Biol. Chem. 267*, 83−90.

Harada, N., & Negishi, M. (1988) *Biochem. Pharmacol. 37*, 4778−4780.

Hasemann, C. A., Kurumabail, R. G., Boddupalli, S. S., Peterson, J. A., and Deisenhofer, J. (1995) *Structure 3*, 41−62.

Hsu, M.-H., Griffin, K. J., Wang, Y., Kemper, B., & Johnson, E. F. (1993) *J. Biol. Chem. 268*, 6939−6944.

Iwasaki, M., Darden, T., Pedersen, L., Davis, D. G., Juvonen, R. O., Sueyoshi, T., & Negishi, M. (1993) *J. Biol. Chem. 268*, 759−762.

Iwasaki, M., Darden, T. A., Parker, C. E., Tomer, K. B., Pedersen, L. G., & Negishi, M. (1994) *J. Biol. Chem. 269*, 9079−9083.

Iwasaki, M., Darden, T. A., Pedersen, L. G., & Negishi, M. (1995a) *Biochemistry 34*, 5054−5059.

Iwasaki, M., Davis, D. G., Darden, T. A., Pedersen, L. G., & Negishi, M. (1995b) *Biochem. J. 306*, 29−33.

Kaipainen, P., Koivusaari, V., & Lang, M. (1985) *Comp. Biochem. Physiol. 81C*, 293−296.

Kedzie, K. M., Balfour, C. A., Escabor, G. Y., Grimm, S. W., He, Y.-A. Pepperl, D. J., Regan, J. W., Stevens, J. C., & Halpert, J. R. (1991) *J. Biol. Chem. 266*, 22515−22521.

Lambert, M. H. (1996) in *Practical Applicaions of Computer-Aided Drug Design*. (Charifson, P., Ed.) Marcel Decker, in press.

Lindberg, R. L. P., & Negishi, M. (1989) *Nature 339*, 632−634.

Lindberg, R. L. P., Juvonen, R., & Negishi, M. (1992) *Pharmocgenetics 2*, 32−37.

Luo, Z., He, Y., & Halpert, J. R. (1994) *Arch. Biochem. Biophys. 309*, 52−57.

Maple, J. R., Hwang, M.-J., Stockfish, T. P., Dinur, U., Waldman, M., Ewig, C. S., & Hagler, A. T. (1994) *J. Comput. Chem. 15*, 162−182.

Negishi, M., Lindberg, R., Burkhart, B., Ichikawa, T., Honkakosi, P., & Lang, M. (1989) *Biochemistry 28*, 4169−6471.

Negishi, M., Uno, T., Darden, T., Sueyoshi, T., & Pedersen, L. (1996) *FESEB J. 10*, 683−689.

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

Ooi, T., Oobatake, M., Nemethy, G., & Scheraga, H. A. (1987) *Proc. Natl. Acad. Sci. 84*, 3086−3090.

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

Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195*, 687−700.

Richardson, T. H., & Johnson, E. F. (1994) *J. Biol. Chem. 269*, 23937−23943.

Straub, P., Johnson, E. F., & Kemper, B. (1993) *Arch. Biochem. Biophys. 306*, 521−527.

Sueyoshi, T., Park, J. L., Moore, R., Juvonen, R. O., & Negishi, M. (1996) *Arch. Biochem. Biophys. 322*, 265−271.

Vasquez, M., & Sceraga, H. A. (1985) *Biopolymer. 24*, 1437−1447.

BI962654J