Altering the Regiospecificity of Androstenedione Hydroxylase Activity in P450s 2a-4/5 by a Mutation of the Residue at Position 481

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ABSTRACT: Mouse P450 2a-5 (coumarin 7-hydroxylase) acquires androstenedione (AD) hydroxylase activity by substituting Phe at position 209 with Asn. However, this mutant P450 2a-5 (F209N) and the corresponding mutant P450 2a-4 (L209N) exhibit different regiospecificites of androstenedione (AD) hydroxylase activity. While the former mutant catalyzes both AD 15α- and 7α-hydroxylase activities at similar rates, the latter mutant maintains the original high specificity of AD 15α-hydroxylase activity. The AD hydroxylase activities in chimeric enzymes of the mutants L209N and F209N show that the regiospecificities are determined by the carboxy-terminal halves of the P450 molecules. Mutations at each of the four different residues within the carboxy-terminal halves indicate that the differences in regiospecificity are determined by the Val/Ala mutation at position 481. As the size of the hydrophobic amino acid at position 481 becomes larger (Ala < Val < Ile), the regiospecificities toward the C15 position of the AD molecule are dramatically increased. The regiospecificity is also increased by placing positively-charged Arg at position 481, although the remaining 15α-hydroxylase activity in this mutant is considerably lower than the other P450s. The results indicate that the size of the residue at position 481 is a key factor in regulating the regiospecificity of AD hydroxylase activity in the P450s. Modeling AD in the substrate-heme pocket of bacterial P450 101A provided further support that residue 481 may reside near the steroid molecule so as to possibly affect the AD hydroxylase activity.

P450 represents a large group of the structurally-related heme-thiolate oxygenases, enzymes that catalyze the oxidations of a vast number of endogenous and exogenous chemicals. Although P450s generally exhibit "broad" substrate specificity, some P450s demonstrate high specificity toward certain substrates. Various hepatic P450s are steroid hydroxylases that exhibit regio- and/or stereospecificities depending on the type of steroid substrates. It is now known that the specificities are determined by the types of a few residues at critical positions in P450s (Lindberg & Negishi, 1989; Negishi et al., 1992; Waterman, 1992; Johnson, 1992). These critical amino acids include residues 113 and 364 in rabbit P450s 2C1 and 2C3, respectively (Kronbach et al., 1991; Hsu et al., 1993); residues 114, 206, 363, and 487 in rat P450 2B1 (Aoyama et al., 1989; Kedzie et al., 1991; He et al., 1992; Halpert & He, 1993; Luo et al., 1994); and residues 117, 209, and 365 in mouse P450s 2a-4/5 (Lindberg & Negishi, 1989; Juvonen et al., 1991; Iwasaki et al., 1992, 1993a). Residues 113, 114, and 117 may correspond in rabbit, rat, and mouse P450s, respectively, as may also residues 364, 363, and 365, respectively. Similarly, the residues at positions 206 and 209 may be homologous in the rat and mouse P450s 2B1 and 2a-4/5, respectively. Despite the diversity in their amino acid sequences, the critical residues appear to be conserved at the corresponding positions in many different mammalian P450s.

P450 2a-4 and P450 2a-5 are members of the mouse 2A subfamily: the former P450 hydroxylates at the 15α-position of Δ^4-3-ketosteroids including testosterone and androstenedione, while the latter is coumarin 7-hydroxylase with little steroid hydroxylase activity (Lindberg & Negishi, 1989). In our previous work (Iwasaki et al., 1994), we found that mutant P450 2a-4 (L209N) maintains its high regiospecificity at the C15 position of the AD molecule, whereas mutant P450 2a-5 (F209N) acquires AD hydroxylase activity but hydroxylates both C15 and C7 positions at similar levels. The present paper extends this finding of the different regiospecificities to identify an amino acid which plays the critical role in the determination of the regiospecificity of AD hydroxylase activity in the P450s 2a-4/5. Moreover, the amino acid sequence of P450 2a-5 is aligned with the bacterial P450cam to locate this critical position in the substrate pocket.

EXPERIMENTAL PROCEDURES

Construction of Chimeric and Mutant P450s. Chimeric P450s (1 and 2) and mutants P450 2a-4 (L209N) and P450 2a-5 (F209N) were constructed as described previously (Iwasaki et al., 1994). Site-directed mutagenesis was performed by using pSELECT vector and the provided protocol (Promega, Madison, WI). The following oligonucleotides were used to mutate Val at position 481: 5′-TGGGATCGTGGCAAAGCC (V481A), 5′-TGGGATCGTGCCAAAGCC (V481G), 5′-TGGGATCGTGATAAAGCC (V481I), and 5′-TGGGATCGTGCGAAAGCCCACGAC

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(V481R). Oligonucleotides used to mutate residues at positions 320, 365, and 426 of P450 2a-5 to the corresponding residues in P450 2a-4 were 5′-ATCTGGGTACTTCATGAG (H320Y), 5′-GGGGATCAGGTCTGCAAA (M365L), 5′-AAAGGATCACTCTTCTT (N426S), and 5′-TGGGATCGTGACAAAGCC (A481V). Underlines indicate the mutated codons. The mutated P450s were confirmed by sequencing the double-stranded templates using a Sequenase kit (UBS, Cleveland, OH).

Expression and Purification. Chimeric and mutated cDNAs were ligated to pAAH5 vectors and transformed into *Saccharomyces cerevisiae* AH22 cells (Iwasaki et al., 1991). Recombinant yeast cells were harvested from 10 L of culture. Then yeast microsomes were prepared was solublized with 100 mM potassium phosphate buffer, pH 7.25, containing 0.4% cholic acid, 0.05% CHAPS, 1 mM DTT, 1 mM EDTA, and 20% glycerol. To obtain purified P450 fractions, solubilized microsomes were applied first to aminooctyl-Sepharose and then to hydroxyapatite columns as described in previous papers (Iwasaki et al., 1991; Juvonen et al., 1993).

Analytical Methods. P450 content was measured following the method of Omura and Sato (1967). Androsterone hydroxylase activity was determined by a previously reported method (Harada & Negishi, 1988). The reaction mixture (0.5 mL) employed 100 mM potassium phosphate buffer, pH. 7.5, and contained P450 (50 pmol), NADPH−P450 reductase (250 pmol), $MgCl_2$ (5 mM), dilauroylphosphatidylcholine (25 μg), [4-^{14}C]androstenedione (100 μM, 50 mCi/mmol), and NADPH (5 mM). The mixture was incubated for 10 min at 37 °C. Steroid metabolites were extracted by methylene chloride and separated by thin-layer chromatography using benzene/chloroform (75:25) as solvent. To calculate the hydroxylase activities, the radioactive bands on TLC plates were scraped and their radioactivities were measured by scintillation counting.

Homology−Function Alignment and Molecular Modeling of AD in the Pocket of P450cam. We modified the algorithm of Smith and Waterman (1981) in order to align the amino acid sequence of P450cam (let it be a_1, a_2, ..., a_m) with that of P450 2a-5 (let it be b_1, b_2, ..., b_n) using additional information about the secondary structure in P450cam and possible locations of selected residues in P450 2a-5. Following Smith and Waterman (1981), we maximized a quantity H_{ij} over $i = 0, ..., m$ and $j = 0, ..., n$, where H_{ij} is interpreted as the maximum similarity of subsequences of P450cam and P450 2a-5 ending in i and j. The Smith−Waterman algorithm depends on two types of parameters. The first type is a residue similarity measure for a pair of residues a and b, $S(a, b)$. The usual choice for S is the standard Dayhoff table. We used a modified measure $S'(a, b)$ defined as follows. Most residue pairs have $S'(a, b) = S(a, b)$. If, however, a residue b_k (for example, residue 209) in P450 2a-5 is hypothesized to match some residue a_i in the substrate-heme pocket of P450cam, then the score for the pair a_i with b_k is $S'(a_i, b_k) = S(a_i, b_k) + \text{BIG}$. BIG is a large positive constant (we used 100), which enforces the constraint that residue b_k is located in the pocket. Based on our site-directed mutagenesis studies, residues 117, 209, 365, and 481 of P450 2a-5 were constrained in this way to land in the pocket of P450cam, by defining the P450cam pocket residues as those with their a-carbons less than 13 Å from the activating oxygen at the 6th axial position of heme in P450cam. The second type of parameter is the Gap initiation and extension penalties. For the most part, we used the defaults (3.0 and 0.1, respectively) from the GAP module from the GCG programs. However, we raised these to 90.0 and 3.0 for insertions or deletions occurring in the conserved helix I in P450cam. We also used the Gotoh recursion method (Gotoh, 1982) to speed the alignment with Gap penalties. Finally, we applied the standard dynamic programming method as described by Smith and Waterman (1981) to maximize the quantity H, and used their traceback procedure to find the sequence alignment. To place the AD molecule in the pocket of P450cam, we followed the procedures of our previous work (Iwasaki et al., 1993a). AD was modeled into the substrate pocket (Darden et al., 1991) and energy-minimized in a two-step procedure using AMBER (Weiner et al., 1984). The pocket atoms were initially relaxed followed by the relaxation of all protein-heme atoms. In the following steps, V396 of P450cam was "mutated" to Ile and then to Ala. Energy-minimization was performed for each of these mutants.

Table 1: Androstenedione Hydroxylase Activity of Mutant P450s[a]

	AA at position 481	androstenedione hydroxylase act. [nmol min^{-1} (nmol of P450)$^{-1}$]		
		15α-OH	7α-OH	15α/7α
single mutation				
P450 2a-5 F209N	Ala	3.28 ± 0.04	1.75 ± 0.1	1.9
P450 2a-4 L209N	Val	8.39 ± 0.07	0.35 ± 0.01	24.0
chimera				
chimera 1	Ala	0.76 ± 0.36	0.28 ± 0.37	2.7
chimera 2	Val	23.4 ± 0.8	0.31 ± 0.04	76
double mutations				
P450 2a-5				
F209N/H320Y	Ala	2.83 ± 0.07	1.52 ± 0.11	1.86
F209N/M365L	Ala	4.22 ± 0.19	1.87 ± 0.12	2.26
F209N/N426S	Ala	3.18 ± 0.1	1.97 ± 0.02	1.61
F209N/A481V	Val	22.4 ± 3.4	0.22 ± 0.04	100.9
F209N/A481I	Ile	11.6 ± 2.4	0.03 ± 0.01	400.0
F209N/A481R	Arg	0.60 ± 0.08	nd[b]	
F209N/A481G	Gly	0.79 ± 0.02	0.05 ± 0.004	15.4
P450 2a-4				
L209N/V481A	Ala	1.56 ± 0.23	1.56 ± 0.14	1.0
L209N/V481I	Ile	3.13 ± 0.42	0.07 ± 0.01	42.9
L209N/V481R	Arg	0.38 ± 0.01	nd	
L209N/V481G	Gly	0.42 ± 0.003	0.12 ± 0.001	3.5

[a] Androstenedione hydroxylase activity was measured as described under Experimental Procedures. [b] nd, not detectable.

RESULTS AND DISCUSSION

Steroid 15α-hydroxylase P450 2a-4 exhibits high regiospecificity at the C15 position of the AD molecule. Mutation of Leu-209 to Asn [P450 2a-4 (L209N)] slightly decreases the specific activity but maintains the high regiospecificity (Table 1). Although coumarin 7-hydroxylase P450 2a-5 shows little steroid hydroxylase activity, a mutant P450 2a-5 (F209N) exhibits the AD 15α- and 7α-hydroxylase activities at similar levels (Table 1). As a result, both mutants P450 2a-4 (L209N) and P450 2a-5 (F209N) catalyze AD hydroxylase activity, yet they exhibit different regiospecificity. To identify which 1 of the 10 different residues between the 2 P450 mutants determines the high regiospecificity at the C15 position of AD molecule, we initially constructed chimeric enzymes between the 2 P450 mutants and measured their AD hydroxylase activities. The convenient *Bam*HI site in the P450 cDNAs was used for the constructions: the 5′-fragment contains 259 amino acid

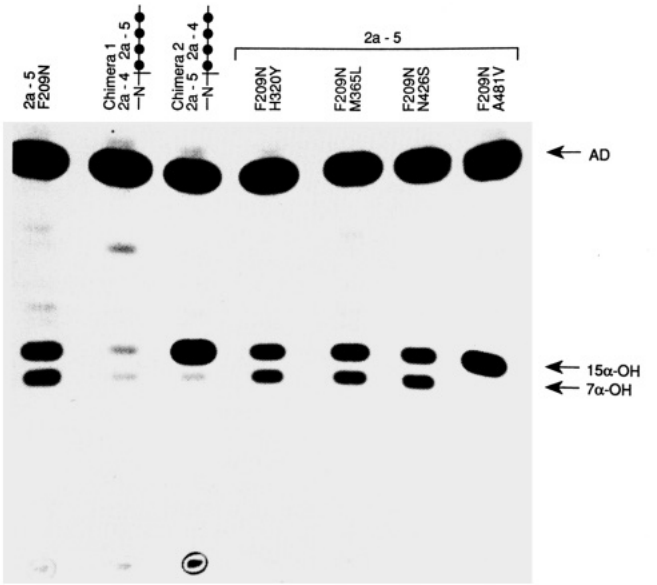

FIGURE 1: AD hydroxylase activities of mutants P450 2a-4 and P450 2a-5. The activities of P450s were reconstituted as described under Experimental Procedures. Steroid metabolites were extracted and analyzed by thin-layer chromatography. One-letter symbols are used to represent the amino acid residues: phenylalanine (F), asparagine (N), histidine (H), tyrosine (Y), methionine (M), leucine (L), alanine (A), valine (V) and serine (S). Closed circles indicate the different residues at positions 320, 365, 426, and 481 in the carboxy-terminal halves.

residues, while the 3′-fragment has 235 residues. Chimera 1, which consists of a N-terminal half of P450 2a-4 (L209N) and a C-terminal half of P450 2a-5 (F209N), catalyzes both AD 15α- and 7α-hydroxylase activities at low but similar levels. Chimera 1 therefore exhibited low regiospecificity as did P450 2a-5 (F209N). The reverse chimera 2, for which the amino- and carboxy-terminal halves were derived from the F209N and L209N mutants, respectively, catalyzed high 15α- and low 7α-hydroxylase activities with the production of approximately 8 times more 15αOH than 7αOH AD (Table 1 and Figure 1). Chimera 2 therefore exhibited a high level of regiospecificity at the C15 position, although the level was somewhat higher than that of the mutant P450 2a-4 (L209N). The results indicate that the differences in the regiospecificity are determined by the carboxy-terminal rather than amino-terminal halves of the chimeric P450s. The carboxy-terminal halves contain different amino acid residues at positions 320, 365, 426, and 481 in the P450s.

Subsequently, we mutated each of the four different residues in the carboxy-terminal half of P450 2a-5 (F209N) to the corresponding one in P450 2a-4 (L209N) and measured the AD hydroxylase activities of those mutated P450s (Figure 1 and Table 1). When Ala-481 was mutated to Val, the resulting mutant P450 2a-5 (F209N/A481V) increased AD 15α-hydroxylase activity, while it decreased AD 7α-hydroxylase activity. This mutant F209N/A481V increased the specific 15α-hydroxylase activity and regiospecificity 3−5-fold compared with those of P450 2a-4 (L209N). Replacing Ala with Val at position 481, therefore, conferred high regiospecificity to P450 2a-5 (F209N) for the C15 position of the AD molecule. On the other hand, the mutations of residues at positions 320, 365, or 426 did not alter either specific AD hydroxylase activities or regiospecificities. We conclude that a single mutation of the residue at position 481 is sufficient to provide P450 2a-5 with the high

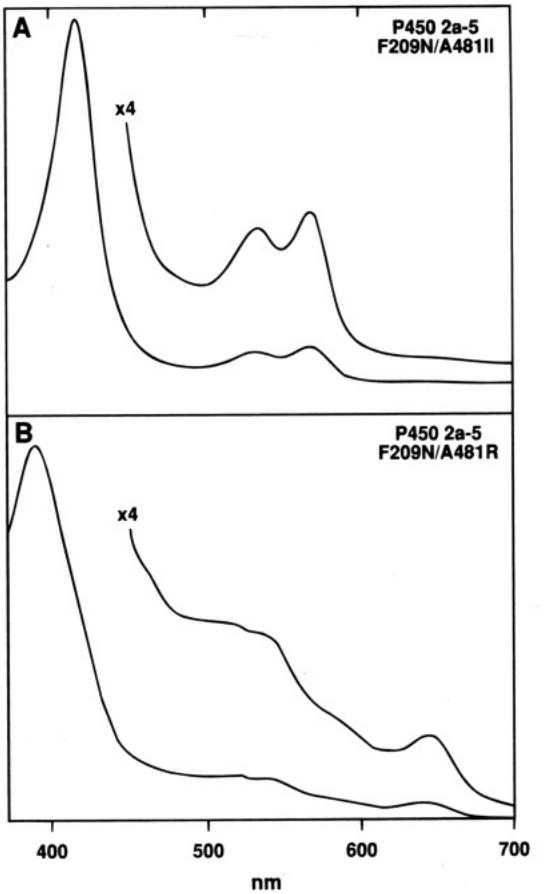

FIGURE 2: Spin state altered by the identity of the residue at position 481. Absolute absorption spectra of the oxidized P450s (approximately 2.0 μM each) were obtained in 400 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.5% sodium cholate, 1 mM EDTA, and 1 mM DTT.

regiospecificity at the C15 position of AD molecule. Consistent with this conclusion, P450 2a-4 (L209N) lost its high regiospecificity by a mutation of Val at position 481 to Ala. Thus, the type of residue at position 481 is apparently the most critical factor for determining the regiospecificity of AD hydroxylase activity in the P450s.

As the size of the residue at position 209 becomes larger, the coumarin 7-hydroxylase activity of P450 2a-5 is increased (Juvonen et al., 1991). The spin equilibrium of P450 2a-4 and P450 2a-5 shifts more toward the high-spin state when a larger amino acid is substituted at residue 209 (Iwasaki et al., 1991). Halpert and his associates have also suggested that the size of residue 478 is a critical factor for the determination of the stereospecificity of AD hydroxylase activity in P450 2B1 (Halpert & He, 1992). Consequently, we examined whether the size of residue 481 is also a critical factor for the determination of the regiospecificity of the AD hydroxylase activity (Table 1). The production ratios of 15αOH and 7αOH ADs by the P450 2a-4 mutants were increased from 1 to 24 and then to 42 by the mutation of residue 481 from Ala to Val, and then to Ile. Similarly, the P450 2a-5 mutants increased the corresponding ratios from 1.9 to 100, and then to 400. The regiospecificity of the AD hydroxylase activity was increased at the C15 position of AD as the size of the hydrophobic residue at position 481 became larger. This role of size was observed even when a charged amino acid was placed at position 481. For instance, when arginine was substituted for position 481, the AD 7α-

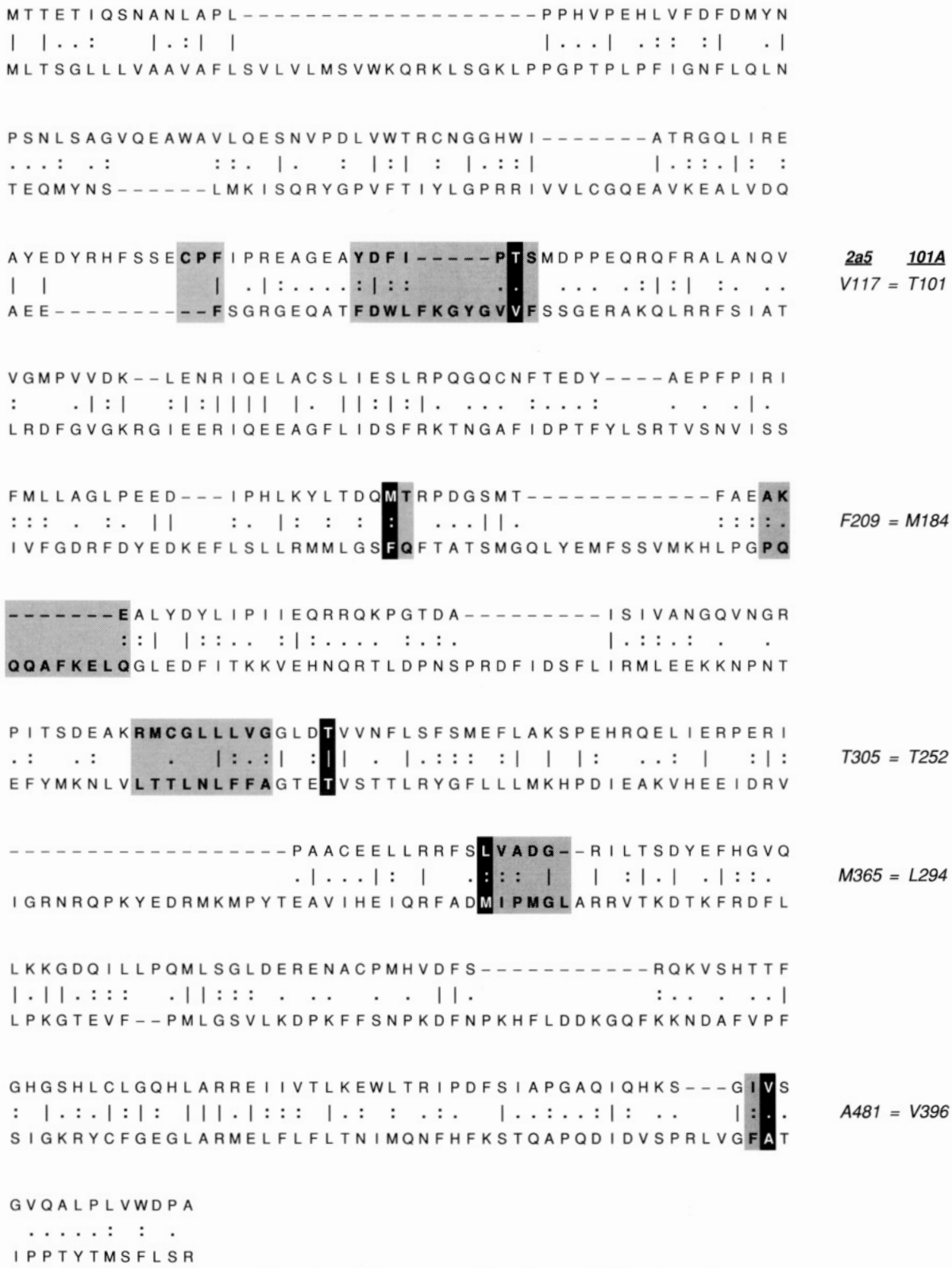

FIGURE 3: Homology−function alignment of the amino acid sequences. The substrate-binding sites of the bacterial P450cam 101A and their corresponding regions of the mouse P450 2a-5 are boxed. The four critical residues and the conserved threonine are darkened and also shown on the right side of the alignment.

hydroxylase activity of both P450 2a-4 and P450 2a-5 was abolished. In addition, the 15α-hydroxylase activity was decreased (Table 1). As a result, the P450s having Arg at position 481 hydroxylated only the C15 position of AD; i.e., the regiospecificity became even higher than that of the corresponding Ile mutants. Given the caveat that only a limited number of amino acids were tested in the present experiments, it appears that the size of the residue at position 481 is a key factor for determining the regiospecificity of AD hydroxylase activity in the P450s. It should be noted that the higher regiospecificity at the C15 position did not always result in higher specific 15α-hydroxylase activity. Chimera 2 and P450 2a-5 (F209N/A481V) exhibited the highest 15α-hydroxylase activities, yet their regiospecificities were 4−5 times lower than the highest regiospecificity of P450 2a-5 (F209N/A481I), and 2 times lower activities than

the chimera and P450 2a-5 (F209N/A481V). Thus, the overall AD hydroxylase activity must be determined by residues other than 481, i.e., by those at positions 117, 209, and 365.

Mutant P450 2a-5 (F209N) exhibited a mixed spectrum with both high- and low-spin forms in the ratio of approximately 1:3 (Iwasaki et al., 1991). The spin equilibrium of the mutant P450s was altered as a function of the type of residue at position 481 (Figure 2). When Val at position 481 was substituted with a larger hydrophobic amino acid, Ile, the spin equilibrium of the mutant P450 was shifted more toward the high-spin form. Conversely, the mutant P450 was completely converted to the low-spin form by placing a charged amino acid, Arg, at position 481. This pattern of spin alteration was reminiscent of that observed in mutant P450s that had various substitutions at position 209 (Iwasaki et al., 1991). These observations indicate that residue 481 may reside sufficiently close to the sixth axial position to affect the iron coordination of a water molecule. The amino acids corresponding to position 481 in other mammalian P450s such as rat P450 2B1 have been proposed to be located in the substrate pocket or substrate-access channel (Aoyama et al., 1989; Kedzie et al., 1991). In addition, this residue is located in one of the six substrate recognition sites (SRSs) proposed by Gotoh (1992). The spin alteration induced by the type of residue 481 supports the proposal that 481 is the critical position and further that the residue may be near the sixth axial position of the heme. Unlike the case of the P450 2a-5 mutants, the P450 2a-4 mutants were always low-spin forms regardless of the type of residue at position 481. This implies a subtle topological difference at residue 481 in the P450 2a-4 and P450 2a-5 molecules.

Since the present site-directed mutagenesis studies implicated residue 481 as a key amino acid for determining the regiospecificity of AD hydroxylase and the high−low-spin equilibrium in the P450s, we aligned the amino acid sequence of P450 2a-5 with that of bacterial P450cam (Figure 3). In our homology−function alignment, residue 481 of P450 2a-5 corresponds to Val at position 396 of P450cam. We then used our previously mimimized structure of corticosterone in the P450cam 101A pocket as a starting point for modeling the AD-binding orientation (Figure 4). Remarkably, after the two-step energy minimization, one of the side chain carbon atoms of V396 was only 3.23 Å from CM13 of the AD molecule. The C7 and C15 positions of the AD molecule were 3.87 and 3.27 Å, respectively, from the oxygen in carbon monoxide at the sixth axial position. These structural features of AD binding were more or less maintained when Ile was substituted for Val at position 396. On the other hand, there was much less van der Waals contact between residue 396 and the AD molecule in the V396A mutation. Consequently, the C7 position of AD could approach closer to the sixth axial position when residue 396 is alanine, without the steric conflict presented by valine. Although it is speculative at the present time, this molecular modeling provides a possible explanation for the loss of regiospecificity of the V481A mutant. It remains, however, for the determination of the 3D structure of P450 2a-5 or a similar P450 to account for the fine details of the catalytic differences.

That there is a functional role for amino acids within the carboxy-terminal region of mammalian P450 was first reported by Imai and his co-workers (Uno & Imai, 1989, 1991). They replaced the carboxy-terminal peptide containing 28 amino acid residues of rabbit P450 (2C2) with the corresponding peptide of P450 2C14. P450 2C2 catalyzes laurate (ω-1) hydroxylation, whereas P450 2C14 is specific for testosterone 16α-hydroxylase. This peptide replacement conferred new testosterone 16β-hydroxylase activity to the hybrid P450, which is not catalyzed by either of the original P450s. In addition, Halpert and his co-workers demonstrated that the type of residue at position 478 plays a critical role in defining the specificity of steroid hydroxylase activity in rat P450 2B1 (Kadzie et al., 1991). Residue 478 of rat P450 2B1 corresponds to residue 481 of mouse P450s 2a-4/5. Halpert et al. found that the mutation from Val to Ala at position 478 altered the stereospecificity of P450 2B1 from AD 16β- to 16α-hydroxylations. Moreover, simultaneous mutation of two residues at positions 114 and 478 converted the regiospecificity of P450 2B1 to AD 15α-hydroxylase activity (Halpert & He, 1993). It appears, therefore, that the functional role and topology of residue 481 in mouse P450s 2a-4/5 are conserved in other mammalian P450s. In addition to residue 481, those at positions 117, 209, and 365 also maintain their functional roles and topologies in other mammalian P450s (Lindberg & Negishi, 1989). Johnson and co-workers reported that the types of residues at positions 113 or 364 (homologous to residues 117 and 365, respectively, of P450s 2a-4/5) are critical for progesterone 21- or 6β-hydroxylase activity in rabbit P450s 2C1 or 2C3 (Hsu et al., 1993). Only two amino acid mutations at positions 114 and 206 or positions 114 and 363 were enough to convert the catalytic characteristics of rat P450 2B1 to those of mouse P450 2a-4, although the two P450s are only 52% identical in their amino acid sequences (Halpert & He, 1993; Lou et al., 1994). We, therefore, propose that four amino acid residues (117, 209, 365, and 481) are conserved in the substrate pocket of mammalian P450s and these play key roles in determination of the steroid hydroxylase activity of the P450s.

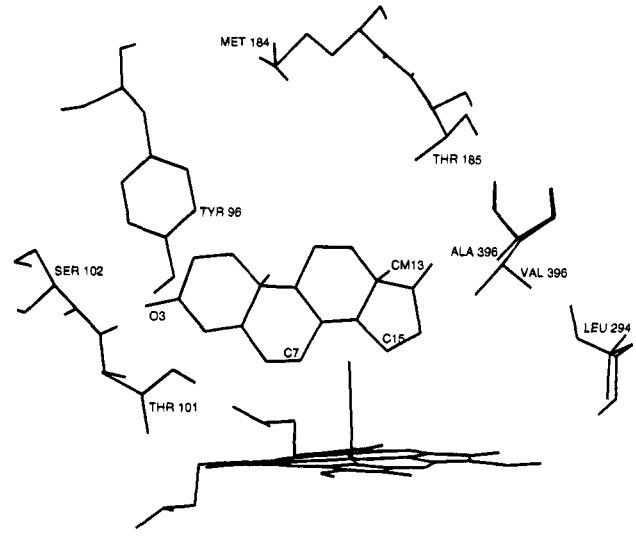

FIGURE 4: Energy-minimized model of AD binding in the pocket of P450cam 101A. In the model, the steroid molecule, heme, and key amino acids are shown. Our homolgy−function alignment suggests that residues 117, 209, 365, and 481 in P450 2a-4 correspond to Thr-101, Met-184, Leu-294, and Val-396, respectively. A geometrical change that occurs by the substitution of Val-396 with alanine is also included. Steroid carbons at positions 7 and 15 are denoted by C7 and C15. O3 designates 3-ketone, while CM13 represents the methyl group at position 13.

In conclusion, the residue at position 481 determines the regiospecificity of AD hydroxylase activities in P450s 2a-4/5. Consequently, residue 481 is another critical amino acid in addition to those at positions 117, 209, and 365 which play a critical role for P450 activity. Our homology–function alignment suggests that residue 481 corresponds to Val at position 396 in the substrate pocket of P450cam. The pocket can accommodate the AD molecule in such a manner that the sixth axial position of the heme is in the close contact with the C15 and C7 positions of the steroid and Val-396 is near CM13.

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