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STRUCTURE-FUNCTION ANALYSIS OF CYP2A10 AND CYP2A11, P450 CYTOCHROMES THAT DIFFER IN ONLY EIGHT AMINO ACIDS BUT HAVE STRIKINGLY DIFFERENT ACTIVITIES TOWARD TESTOSTERONE AND COUMARIN

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SUMMARY: Cytochrome P450 NMa, which was first identified in this laboratory in rabbit nasal microsomes, is now known to represent two distinct gene products, P450s 2A10 and 2A11. In the present study, chimeric and site-directed mutants of 2A11 were constructed to determine which of the eight different amino acid residues are responsible for the much greater activity of 2A10 toward coumarin and testosterone. Mutation of Arg^{62} and Asp^{63} of 2A11 to the corresponding residues in 2A10, or mutation of Thr^{120} to Ser, as found in 2A10, did not change the activities. However, mutation of Arg^{62}, Asp^{63}, Gln^{104}, Ala^{117}, and Thr^{120} of 2A11 to the corresponding residues in 2A10 resulted in a protein that is as active as 2A10 in coumarin hydroxylation and approximately half as active as 2A10 in androstenedione formation. Mutation of Arg^{372} in 2A11 to His, as found in 2A10, resulted in a significant increase in the rate of hydroxylation of testosterone, but not of coumarin. Our findings indicate that the identity of the amino acid at position 104 and/or 117 is important for activity with testosterone and for regioselectivity at the 17 position, as well as for optimal activity with coumarin. In contrast, the identity of the residue at position 372 is important for optimal activity with testosterone but not the regioselectivity at the 17 position and does not influence the activity with coumarin. © 1994 Academic Press, Inc.

Recently, cDNA clones encoding two highly similar P450s, designated 2A10[1] and 2A11, were obtained from a rabbit nasal cDNA library (2). Both 2A10 and 2A11 contain 494 amino acids and correspond to purified P450 NMa (3) in the first 20 amino acids. The predicted amino acid sequences of the two proteins differ in only eight positions and are over 80% identical to the sequences of 2A3, 2A4, 2A5, 2A6, and 2A7 (see Ref. 1 for sequence information). After heterologous expression of 2A10 and 2A11 cDNAs in *Escherichia coli*, both enzymes are active toward several

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ABBREVIATIONS: 2A10 and 2A11, cytochrome P450 isoforms 2A10 and 2A11 (see Ref. 1 for updated P450 nomenclature).

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xenobiotic substrates, although 2A10 is generally more active than 2A11. In the present study, structure-function analysis was carried out to determine which of the eight different amino acid residues contribute to the 10-fold greater activity of 2A10 toward coumarin and more than 20-fold greater activity in the conversion of testosterone to androstenedione. The data obtained suggest that Leu^{104} and Val^{117} are important for the androstenedione formation and coumarin 7-hydroxylation activities of 2A10 and that mutation of $Arg^{372} \rightarrow His$ results in a significant increase in the rate of testosterone hydroxylation by 2A11.

METHODS

Construction and heterologous expression of mutant P450 2A genes. Chimeras of 2A10 and 2A11 were produced using restriction endonuclease sites common to both cDNAs. Chimera A was constructed using *Apa* I, which cleaves at nucleotides 110 and 223, and chimera B with *Bgl* I, which cleaves at nucleotides 105 and 381. Site-directed mutagenesis of 2A11 was carried out by the polymerase chain reaction (PCR) with primers containing the desired mutation and 2A11 cDNA as the template. The PCR fragments were generated in a Perkin-Elmer Cetus DNA thermal cycler using *Taq* DNA polymerase. Each of the 30 thermal cycles included a denaturation step at 94°C for 1 min, an annealing step at 68°C for 1 min, and an extension step at 72°C for 3 min. For the $Thr^{120} \rightarrow Ser$ mutant, the primers used were 5'-AAGCTCTGGGGGAAGCTGCCC-3' (upstream) and 5'-AAGCGCCGCAGC-GGCCTGGCGCGCTCCCAG<u>C</u>TG-3' (downstream), with the underlined residue indicating the nucleotide mutated. The resulting fragment was then digested with *Bgl* I and ligated with similarly digested 2A11 cDNA. The $Arg^{372} \rightarrow His$ mutant was constructed by replacing an *Nco* I/*Bst* E2 fragment in 2A11 cDNA with an *Nco* I/*Bst* E2 linker (5'-CATGGGCCTGGCCC<u>A</u>CAGG-3'/5'-CCGGACCGGG<u>T</u>GTCCCAGTG-3') that contained the mutation. The DNA sequence of the PCR-generated segments was determined to ensure the fidelity of *Taq* DNA polymerase extension of the amplified primers, and all mutations were confirmed by sequence analysis of the resulting DNA constructs. Oligonucleotides were synthesized with automated instruments from Applied Biosystems by the DNA facility at the University of Michigan. *E. coli* strain XL1-blue was transformed with the expression plasmids as described (3,4) and was used for heterologous expression of the recombinant P450s. Partial purification and spectral determination of recombinant P450s from solubilized bacterial membranes were as described previously (3).

Determination of catalytic activity. Procedures for the determination of coumarin and testosterone hydroxylation by recombinant P450s in a

reconstituted system were as described (3). For coumarin 7-hydroxylation, the reaction mixtures contained 50 μmol of potassium phosphate buffer, pH 7.4, 0.05 nmol of P450, 0.2 nmol of NADPH-cytochrome P450 reductase, 30 μg of dilauroylglyceryl-3-phosphorylcholine, 0.5 μmol of coumarin added in 12.5 μl of methanol, and 1 μmol of NADPH as the final addition in a total volume of 1.0 ml. The incubation was carried out at 37°C for lengths of time that represented the initial linear rate of product formation. For testosterone hydroxylation, the reaction mixtures contained 5 nmol of [1,2,6,7-^{3}H(N)]testosterone (4.7 Ci/mmol, added in 10 μl of methanol), 1 μmol of ascorbic acid, and other components as described above.

RESULTS AND DISCUSSION

The distinct substrate specificity of 2A10 and 2A11 toward testosterone and coumarin makes these enzymes of interest for examination of the amino acid residues involved in substrate binding and oxygenation. One of the regions of greatest dissimilarity between these cytochromes is at amino acid residues 117-120; marked dissimilarity in this region is also found between P450 2A1 and 2A2 (5), 2A4 and 2A5 (6,7), 2C4 and 2C5 (8), and 2B1 and 2B2^{V} (9). In the case of P450 2A4 and 2A5, Val^{117} in the former cytochrome appears to be critical for coumarin 7-hydroxylation (10). In addition, the corresponding region in 2C4 and 2C5 (amino acid residues 113-115) was found to be important in progesterone 21-hydroxylation (8). Gotoh (11) has recently proposed six substrate-recognition regions in P450 family 2 proteins, based on group-to-group alignment of CYP2 sequences and those of bacterial P450s, including P450$_{cam}$, or CYP101A, for which substrate-binding residues have been identified by x-ray crystallography (12). Interestingly, as shown in Fig. 1, five of the eight amino acid differences between 2A10 and 2A11 fall into those proposed substrate recognition sites.

Structure-function analysis indicated that not all of the eight different amino acid residues are important for the 10-fold greater activity of 2A10 toward coumarin and more than 20-fold greater activity in the conversion of testosterone to androstenedione in a reconstituted system, as shown in Table I. Mutation of Arg^{62} and Asp^{63} of 2A11 to the corresponding residues in 2A10 (chimera A), or mutation of Thr^{120} to Ser (as found in 2A10) did not change the activities of 2A11 toward coumarin or testosterone, but mutation of Arg^{62}, Asp^{63}, Gln^{104}, Ala^{117}, and Thr^{120} of 2A11 to the corresponding residues in 2A10 (chimera B) resulted in a protein with the same activity as 2A10 in coumarin hydroxylation and approximately half of the activity of 2A10 in androstenedione formation. Thus, at least two of the five residues (Leu^{104} and Val^{117}) located in the

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CYP2A10   MLASGLLLAALLACLTVMILLSVWRQRKLWGKLPPGPTPLPFIGNYLQLN  50
CYP2A11   MLASGLLLAALLACLTVMILLSVWRQRKLWGKLPPGPTPLPFIGNYLQLN  50

CYP2A10   TEQMYDSLMKISERYGPVFTIHLGPRRIVVLCGQEAVKEALVDQAEDFSG 100
CYP2A11   TEQMYDSLMKIRDRYGPVFTIHLGPRRIVVLCGQEAVKEALVDQAEDFSG 100
                     **
CYP2A10   RGELATFDWLFKGYGVVFSSWERARPLRRFAISTLRDFGVGKRGIEERIQ 150
CYP2A11   RGEQATFDWLFKGYGVAFSTWERARPLRRFAISTLRDFGVGKRGIEERIQ 150
             *            *  *
CYP2A10   EEAGFLIEAFRDTRGAFIDPTFFLSRTVSNVISSIVFGDRFDYEDKEFLS 200
CYP2A11   EEAGFLIEAFRDTRGAFIDPTFFLSRTVSNVISSIVFGDRFDYEDKEFLS 200

CYP2A10   LLRMMLGSFQFTATPTGQLYEMFYSVMKHLPGPQQQAFKELEGLRDFIAK 250
CYP2A11   LLRMMLGSFQFTATPTGQLYEMFYSVMKHLPGPQQQAFKELEGLRDFIAK 250

CYP2A10   KVERNQRTLDPNSPRDFIDSFLIRMQEEKKDPKSEFHMKNLVMTTLNLFF 300
CYP2A11   KVERNQRTLDPNSPRDFIDSFLIRMQEEKKDPKSEFHMKNLVLTTLNLFF 300
                                                    *
CYP2A10   AGTETVSTTMRYGFLLLMKHPDVEAKVHEEIDRVIGRNRQPKFEDRAKMP 350
CYP2A11   AGTETVSTTMRYGFLLLMKHPDVEAKVHEEIDRVIGRNRQPKFEDRAKMP 350

CYP2A10   YTEAVIHEIQRFTDMIPMGLAHRVTRDTKFRDFLLPKGAEVFPMLGSVLK 400
CYP2A11   YTEAVIHEIQRFTDMIPMGLARRVTRDTKFRDFLLPKGTEVFPMLGSVLK 400
                               *                *
CYP2A10   DPKFFSKPREFYPQHFLDEKGQFKKSDAFMPFSVGKRYCLGEGLARMELF 450
CYP2A11   DPKFFSKPREFYPQHFLDEKGQFKKSDAFMPFSVGKRYCLGEGLARMELF 450

CYP2A10   LFFTTIMQNFRFRSQQAPQDIDVSPKHVGFATIPRTYTMSFVPR     494
CYP2A11   LFFTTIMQNFRFRSQQAPQDIDVSPKHVGFATIPRTYTMSFVPR     494
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Fig. 1. Sequence alignment of P450 2A10 and 2A11. The positions of eight amino acid differences are indicated by an *asterisk*, and the underlined areas are the substrate recognition sites proposed by Gotoh (11).

proposed substrate-recognition regions may be involved in binding with coumarin and testosterone. Additional studies are in progress with Gln^{104}→Leu and Ala^{117}→Val mutants of 2A11 to determine whether one or both of the amino acids at these positions are responsible for the higher

Table I. Catalytic activities of wild-type and mutant P450 2A10 and 2A11 cytochromes[a]

Enzyme	Amino Acid No.								Coumarin hydroxylase	Testosterone hydroxylase		
	62	63	104	117	120	293	372	389		X1+15α	11β	17
									nmol/min/nmol	*pmol/min/nmol*		
2A10	S	E	L	V	S	M	H	A	1.78	34	65	217
2A11	R	D	Q	A	T	L	R	T	0.17	69	22	<10
Chimera A	<u>S</u>	<u>E</u>	Q	A	T	L	R	T	0.18	61	17	<10
Chimera B	<u>S</u>	<u>E</u>	<u>L</u>	<u>V</u>	<u>S</u>	L	R	T	1.80	17	13	95
Thr^{120}→Ser	R	D	Q	A	<u>S</u>	L	R	T	0.18	58	32	<10

[a]The values reported are the average of duplicate determinations, with a standard error less than 10% of the mean. The specific content of P450 for the partially purified recombinant proteins ranged from 1.5 to 3.0 nmol/mg of protein. The underlined residues correspond to those of 2A10. X1 designates an unidentified metabolite of testosterone, and 15α, 11β, and 17 indicate 15α- and 11β-hydroxytestosterone and androstenedione, respectively. X1 and 15α-hydroxytestosterone, which were not completely resolved, were determined together.

activities of 2A10 in coumarin hydroxylation and androstenedione formation.

Interestingly, mutation of Arg^{372} in 2A11 to His (as found in 2A10) resulted in an increase in the rates of formation of all testosterone metabolites, but gave the same metabolite profile as that of 2A11 (Table II). However, the $Arg^{372}{\rightarrow}His$ mutation did not result in a significant increase in coumarin hydroxylation activity (data not shown). In other experiments not presented, both 2A11 and the $Arg^{372}{\rightarrow}His$ mutant showed higher activity toward testosterone at pH 6.7 than at pH 7.4, with the mutant cytochrome being more active than the wild type at either pH. Furthermore, the difference in turnover number toward testosterone appears to be due to intrinsic properties of the P450 proteins, not to other components in the partially purified enzyme preparations. This was demonstrated by the finding that, when 2A11 and the $Arg^{372}{\rightarrow}His$ mutant were mixed before reconstitution with P450 reductase and phospholipid, the combined activity was equal to the sum of the activities of the two cytochromes determined individually (not shown). Thus, it appears that, although the residue at position 372 is not involved in determining the regiospecificity of testosterone hydroxylation by 2A10 and 2A11, it is important for optimal overall activity.

It remains to be determined whether the increase in testosterone hydroxylase activity is a result of altered substrate binding or an enhanced rate of electron transfer or oxygen insertion. According to the sequence alignment proposed by Gotoh (11), Arg^{372} in 2A11 corresponds to Arg^{299}

Table II. Testosterone hydroxylation by 2A10, 2A11, and the $Arg^{372}{\rightarrow}His$ mutant of 2A11[a]

Testosterone concentration	Enzyme	X1+15α	11ß	17
			pmol/min/nmol	
5 μM	2A10, wild-type (n=8)	19 ± 7	35 ± 16	166 ± 38
	2A11, wild-type (n=12)	60 ± 16	27 ± 9	< 5
	2A11, $Arg^{372}{\rightarrow}His$ (n=12)	111 ± 22	61 ± 16	10 ± 4
25 μM	2A11, wild-type (n=2)	166 ± 43	74 ± 2	<30
	2A11, $Arg^{372}{\rightarrow}His$ (n=2)	280 ± 4	155 ± 11	<30

[a]The assay conditions were the same as described in Table I except that a different batch of P450 reductase, which had lower activity, was used. The values reported represent the mean ± standard error, with the number of experiments performed (n) indicated in parentheses. The low levels of androstenedione formed by 2A11 and its mutant could not be determined accurately due to interference from a contaminant with a similar retention time.

in $P450_{cam}$, which is part of the β3 strand; this strand contacts both the heme and the substrate (12). Sequence alignment of $P450_{BM-3}$ and $P450_{cam}$ based on their tertiary structures indicated that the β strand structure corresponding to β3 in $P450_{cam}$ also occurs in $P450_{BM-3}$ and contributes to the substrate-binding pocket (13). Hasemann *et al.* (14) recently suggested that Arg^{319} in $P450_{terp}$, which corresponds to Arg^{299} in $P450_{cam}$ based on alignment of their tertiary structures, may also be involved in interaction with the oxygen of the heme propionate through hydrogen bonding with an intervening water molecule. Thus, as pointed out by Poulos (15), mutation in this region may affect heme binding as well as substrate interaction.

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