Site-Directed Mutagenesis of Cytochrome P450s CYP2A1 and CYP2A2: Influence of the Distal Helix on the Kinetics of Testosterone Hydroxylation

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ABSTRACT: Cytochrome P450s CYP2A1 and CYP2A2 exhibit 88% sequence similarity, yet CYP2A1 metabolizes testosterone almost exclusively (90%) at the 7α-position, whereas CYP2A2 forms several metabolites, with 15α-hydroxytestosterone as a major metabolite. One of the regions with relatively low sequence homology corresponds by sequence alignment to the I and J helices of P450cam. Since this region is known to be part of the active site for P450cam, 26 single point and two double point mutants were prepared where the amino acid for one form was substituted with that of the other. Mutant and wild-type enzymes were expressed in Hep G2 cells using the vaccinia virus vector. Analysis of testosterone regioselectivity revealed that 25 of the mutants show the same regioselectivity as the parent wild-type enzymes and three are inactive, suggesting that no single amino acid in this region is totally responsible for the different selectivities of CYP2A1 and CYP2A2. Kinetic analysis of the CYP2A1 mutants showed that four of the mutants with changes near the conserved oxygen-binding region had K_m values with much higher and V_{max} values much lower than those of the wild-type enzyme and one mutant had a V_{max} value twice as high as that of the wild-type enzyme. Deuterium isotope effects on 7α-hydroxxylation were used to determine changes in the rate of reduction and estimate the relative amount of excess water formation. Changes in reduction rates and the amount of water produced are not sufficient to account for the differences in V_{max} values, suggesting that the amount of hydrogen peroxide released is a primary determinant for changes in V_{max}.

The cytochrome P450 enzymes are a superfamily of monooxygenases which have adopted diverse roles in oxidative metabolism. The common catalytic function of these enzymes is the two-electron reduction of molecular oxygen to form water and a reactive oxygen species. This reactive oxygen is used in both the metabolism of endogenous compounds such as steroids and prostaglandins and in the metabolism of exogenous chemicals such as drugs and environmental contaminants (Ortiz de Montellano, 1986; Guengrich, 1987). Since the only cytochrome P450 crystal structure available is that for bacterial P450cam (Poulos et al., 1987), very little is known about the three-dimensional structure of the mammalian P450s and thus their catalytic and substrate recognition sites. Recent cloning and sequencing of numerous cytochrome P450s (Gonzalez, 1989; Nebert et al., 1991) together with secondary structure predictions (Nelson & Strobel, 1989; Gotoh & Fujii-Kuriyama, 1989) have suggested that many of the secondary structural elements have been conserved. Construction of P450 chimeras and site-directed mutagenesis in conjunction with cDNA expression have been used to provide structural information on substrate-binding sites (Kronbach, et al., 1989; Lindberg & Negishi, 1989; Uno & Imai, 1989; Aoyama et al., 1989; Shimizu et al., 1989), NADPH-P450 oxidoreductase binding sites (Shimizu et al., 1989), and other properties (Shimizu et al., 1988; Lorence et al., 1990; Iwasaki et al., 1991).

In a previous study from our laboratory, chimeras were constructed from 2A1 and 2A2 cDNAs and expressed using a vaccinia virus vector (Hanioka et al., 1990). The wild-type enzymes, although having 88% sequence homology, have very different regioselectivities for testosterone hydroxylation. Cytochrome P450 2A1 metabolizes testosterone almost exclusively (90%) at the 7α-position, whereas P450 2A2 has a much broader specificity, forming many metabolites including 15α-, 12α-, 7α-, and 6β-hydroxytestosterones. The regioselectivity observed for metabolism of testosterone with the chimeric forms suggested that both specific and nonspecific binding interactions were involved with both isozymes. The results suggested that the region between amino acids 275 and 355 was important in maintaining regioselectivity. Sequence alignment and secondary structure predictions for these enzymes indicate that this region contains helices corresponding to the I and J helices of P450cam. The I helix of P450cam passes directly over the heme and is part of the substrate-binding region. This helix is one of the most conserved secondary structures, and contains a conserved region which is thought to provide an oxygen-binding site (Poulos et al., 1987). Since there are 13 amino acid differences between P450 2A1 and 2A2 in the region between 279 and 331, 26 single point and two double point mutants were prepared where the amino acid for one isozyme was substituted with that of the other. The preparation and kinetic characterization of these mutants is the subject of this report.

Experimental Procedures

Materials. Testosterone and 11α-hydroxyprogesterone were obtained from Steraloids. [4-^{14}C]Testosterone was purchased from Amersham Corp. Synthesis of 6α,7α-$^2H_2$-testosterone has been described previously (Korzekwa et al., 1990).

Site-Directed Mutagenesis and Construction of Recombinant Vaccinia Viruses. The 2A1 and 2A2 cDNAs were inserted into the *Eco*R1 site of M13 mp8. 17-mer or 18-mer oligonucleotides containing single or double base changes for amino acid codon changes were synthesized using an Applied Biosystems model 380B Synthesizer. The oligonucleotides shown in Figure 1 were not purified. For the double mutants, a second oligonucleotide was used to produce another amino

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P450 2A1 Oligonucleotide		P450 2A2 Oligonucleotide	
A1-01	Asn279Tyr 3' ATTGCCATATTTCTCCT 5'	A2-01	Tyr279Asn 3' ATTAACATTTTTCTCCT 5'
A1-02	Gly280Val CTGAATTGACATTTTTC	A2-02	Val280Gly CTGAATTACCATATTTC
A1-03	Lys287Asn ACTAGGTTGTTCATGTG	A2-03	Asn287Lys ACTAGGTTCTTCATGTG
A1-04	Thr292Ser TAGTGTTGACATCACTA	A2-04	Ser292Thr TAATGATGTCATCACTA
A1-05	Thr293Ser GCTTAGTGATGTCATCA	A2-05	Ser293Thr GCCTAATGTTGACATCA
A1-06	Ser295Gly GAAGAGGCCTAGTGTTG	A2-06	Gly295Ser GAGGAGGCTTAATGATG
A1-07	Phe297Leu AGCAAAGAGGAGGCTTA	A2-07	Leu297Phe AGCAAAGAAGAGGCCTA
A1-08	Ser301Thr TGTCTCAGTCCCAGCAA	A2-08	Thr301Ser TGACCCAGACCCAGCAA
A1-09	Glu302Gly TGACTGTCCCAGACCCA	A2-09	Gly302Glu TGACTGACTCAGTCCCA
A1-10	Thr303Ser GCTGACTGACTCAGACC	A2-10	Ser303Thr GCTGACTGTCCCAGTCC
A1-11	Arg309Tyr AGCCGTAGTATAGTGTGG	A2-11	Tyr309Arg AACCATGGCGTAGCGTGG
A1-12	Tyr310His GAAGCCGTGGCGTAGTG	A2-12	His310Tyr GAAACCATAGTATAGCG
A1-13	Gln331Arg CGATCACCGCTCAAATT	A2-13	Arg331Gln CGATCACTTGCTCAATT

FIGURE 1: Oligonucleotides used in the preparation of the mutant used in the mutagenesis and construction of recombinant vaccinia viruses (see Experimental Procedures).

acid codon change in a single mutant. The mutagenesis was carried out using a kit supplied by Amersham Corp. Six plaques were recovered from each oligonucleotide-directed experimental sample and sequenced using the dideoxynucleotide chain termination procedure. Usually four to six mutants were recovered. Double-stranded plasmid was purified from one of the positive mutants, and an *Sph*I/*Xho*I fragment encompassing amino acid residues between position 275 and 355 of the cDNA was isolated (Hanioka et al., 1990). This was directly inserted into the vaccinia virus transfer plasmid pSC11 containing the normal 2A1 or 2A2 cDNAs. Recombinant vaccinia viruses were synthesized as discussed (Gonzalez et al., 1991).

Analysis of P450 Production from Recombinant Vaccinia Viruses. Viruses were used to infect confluent cultures of Hep G2 cells at a multiplicity of 2 to 5. Cells were harvested and lysed 24 h after infection by brief sonication in 100 mM potassium phosphate buffer, pH 7.4, at a final protein concentration of >10 mg/mL. Expression of P450 was monitored by Western immunoblotting using rabbit anti-rat 2A1 and alkaline phosphatase conjugated goat anti-rabbit IgG. The blots were developed using the BCIP/BNT Phosphatase Substrate System of BRL Laboratories (Gaithersberg, MD). Catalytic activities of the various mutants were first assessed by in situ metabolism of [4-^{14}C]testosterone as described previously (Hanioka et al., 1990), using the TLC procedure of Waxman et al. (1983) to separate testosterone from the hydroxylated metabolites. P450 levels were quantitated by CO-reduced spectral analysis as described by Omura and Sato (1964).

Kinetic Studies. For the K_m–V_{max} studies, cell lysates were prepared as described above and centrifuged at 460000*g* for 10 min. The pellets were collected and resuspended in 50 mM potassium phosphate, pH 7.4, and P450 levels were determined. Each incubation contained 8–40 pmol of P450, 0.15–2 μM testosterone, 1 mM NADPH, and 0.06% methanol in a total volume of 4 mL of buffer (50 mM potassium phosphate, pH 7.4). The incubations were initiated by addition of the NADPH and carried out at 37 °C for 5 min. The reactions were stopped by the addition of 2.5 mL of dichloromethane. For the isotope effect studies, incubations were carried out for 20 min at 37 °C and contained 20–80 pmol of cytochrome P450, 250 μM testosterone, or 6α,7α-$^2H_2$-testosterone, 1 mM NADPH, and 0.12% methanol in a total volume of 1 mL of buffer (50 mM potassium phosphate, pH 7.4). Again, the incubations were initiated by addition of the NADPH and stopped by the addition of 2.5 mL of dichloromethane.

Samples were analyzed by HPLC as described previously (Korzekwa et al., 1990), with the following exceptions: Progesterone (1 μmol) was added to the dichloromethane-incubation mixture to prevent nonspecific binding during the extraction procedure. Testosterone metabolites were separated on a Thomson 20/20 ODS column with a solvent gradient between 85:0:15 and 28:57:15 (water:methanol:tetrahydro-

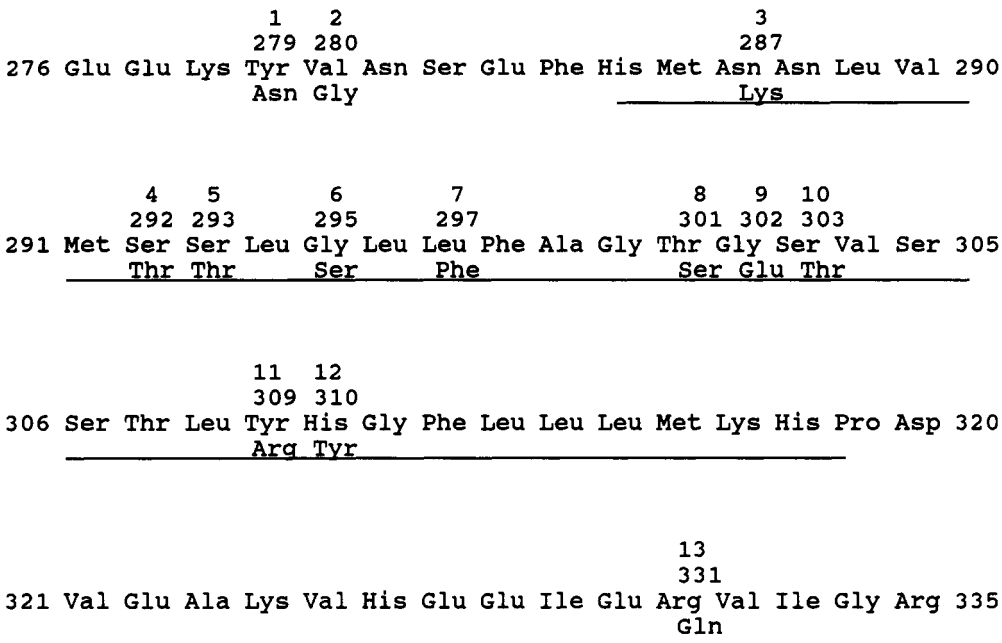


FIGURE 2: Region of the 2A P450s in which the mutations occur. The sequence given is for P450. 2A2 and 2A1 substitutions are shown below. The underlined region is the region which aligns with the I helix of P450cam.

furan) over 17.5 min. The metabolites were quantitated with an HP 1050 UV detector operating at 242 nm by relating peak areas of metabolites to the internal standard, 11α-hydroxyprogesterone. For the K_m–V_{max} studies, incubations at the lowest substrate concentrations were allowed to proceed with up to 50% substrate consumption to increase the sensitivity of the assay. Substrate concentrations were then corrected using the integrated Michaelis–Menton equation. Linear double-reciprocal plots and results independent of total consumption suggest that product inhibition is negligible.

Results and Discussion

In a previous study, analysis of the product selectivity of several chimera of P450s 2A1 and 2A2 suggested that there are both specific and nonspecific binding interactions for both of these enzymes (Hanioka et al., 1990). This was based on the fact that all active chimeras in which less than 276 amino acids correspond to a single isozyme have the same regioselectivity (7α-, 6β-, and 12α-hydroxylation), even diametric opposites. The specificities of the wild-type enzymes were obtained with chimeras containing the first 355 amino acids of the wild-type enzymes, suggesting that the regions of the polypeptide between amino acids 275 and 355 were responsible for the specific interactions. According to previously reported sequence alignments with P450cam (Nelson & Strobel, 1989; Gotoh & Fujii-Kuriyama, 1989), this region includes the I helix, which passes over the heme and contains a conserved oxygen-binding site and makes up part of the active site. Since this region contained 13 amino acid differences between 2A1 and 2A2, each of these differences were exchanged, giving a set of 26 single point mutants. The amino acid sequences for these enzymes and their alignment with the secondary structure of P450cam (Gotoh & Fujii-Kuriyama, 1989) are given in Figure 2. The mutations are labeled 1–13 on the basis of their occurrence in this region. Although mutants 1 and 2 occur in the region which aligns with the β_2 β-sheet of the P450cam sequence, the amino acids in this region are not likely to form a β-sheet (Prevelige & Fasman, 1989). Nelson and Strobel's secondary structure predictions also suggest that this β-sheet is not conserved. Mutants 3–12 have changes in the region which corresponds to the I helix of P450cam. Four of these mutations (4, 5, 8, and 10) are serine–threonine interconversions and three (8, 9, and 10) are in the conserved oxygen-binding region. Mutation 13 aligns with the C-terminal end of the J helix of P450cam.

Autoradiograms of the testosterone metabolites generated in situ from the wild types and mutants are shown in Figure 3. Of the 26 single point mutants and 2 double mutants, 4 are inactive (2A1 mutants 4 and 13 and 2A2 mutants 6 and 12) and 24 are active and show the same regioselectivity of the parent wild-type enzyme. Three of the inactive mutants show a lack of or low level production of P450 protein (Figure 4) which may be due to instability and premature degradation. It is evident that no single amino acid which has been changed and resulted in an active enzyme is totally responsible for the differences in selectivity between 2A1 and 2A2. This leaves two possibilities concerning the binding interactions between the substrate and the region between 275 and 355 which were implied in the previous chimera studies (Hanioka et al., 1990). One possibility is that the amino acids which bind the substrate are those which resulted in the inactive mutants when changed. Of the four inactive mutants, 2A1–13 is likely to be far from the binding site, leaving 2A1–4, 2A2–6, and 2A2–12 which are likely to be in the I helix. Although it is possible that these amino acid residues are both involved in substrate binding and crucial for enzyme stability, it seems more likely that changes in local conformation are involved. It has been proposed previously by Poulos et al. (1987) that the substrate access channel for P450cam are hydrophobic residues which undergo dynamic fluctuations. If the binding sites for mammalian P450s also consist of "flexible" regions in the protein, these regions may be more likely to undergo local conformational changes. The autoradiograms also suggest that there are some differences in rates of product formation. Since the 2A1 isozyme was shown previously to have an unusually low K_m [0.6 μM (Korzekwa et al., 1990)] and high regioselectivity, the 2A1 mutants were studied more closely using kinetic and isotope effect experiments in order to detect any changes in binding and rate constants.

Kinetic analyses of the P450s are complicated by both the multistep nature of the enzymatic cycle and the possibility of multiple pathways (Scheme I). Oxygen activation requires

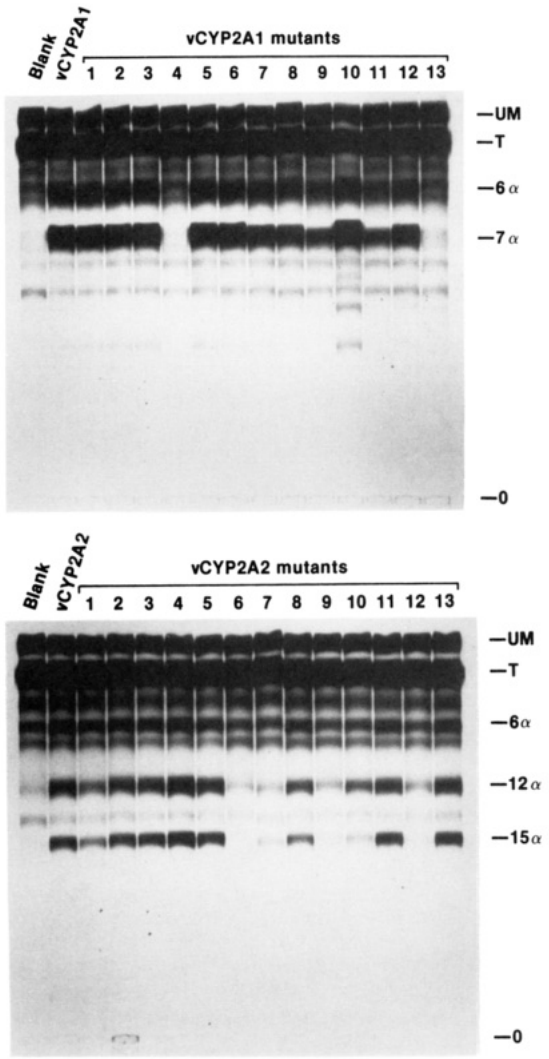
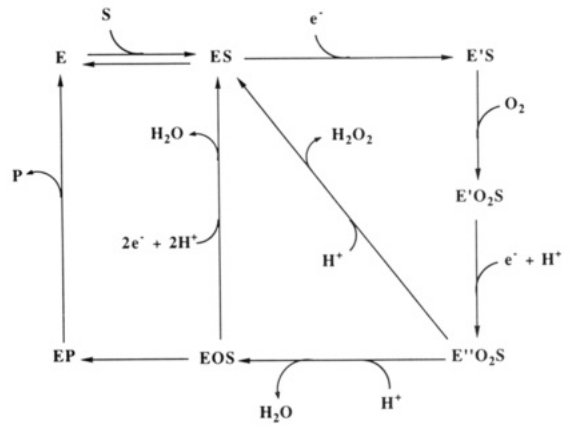


FIGURE 3: Western immunoblots of protein from cells infected with recombinant vaccinia viruses (see Experimental Procedures). (a, top) P450 2A1 mutants; (b, bottom) P450 2A2 mutants.

Scheme I



the introduction of two electrons and two protons and the release of a molecule of water. In addition, two nonproductive pathways have been documented. These are the release of hydrogen peroxide (Gillette et al., 1957; Norblom & Coon, 1982) and the further reduction of the active oxygenating species to water (Morgan et al., 1982; Gorsky et al., 1982). Hydrogen peroxide release may compete with heterolytic cleavage of the dioxygen bond, and the excess water formation has been shown to be in competition with substrate oxidation (Atkins & Sligar, 1987).

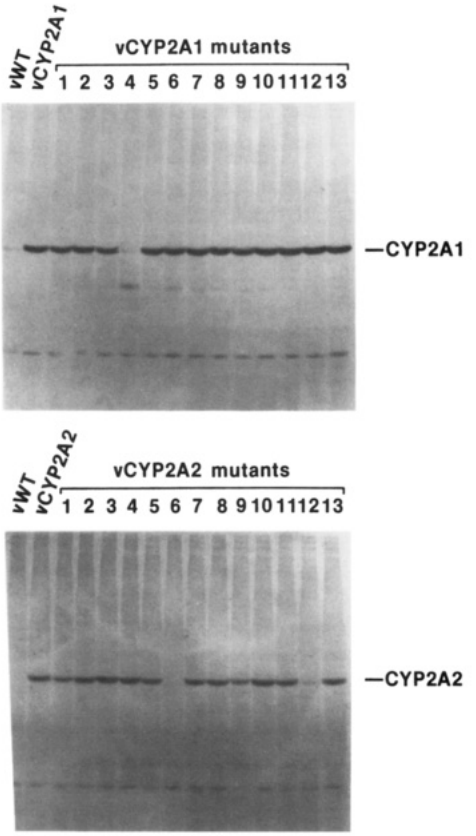


FIGURE 4: Autoradiograms of the TLC separations of testosterone and metabolites generated in situ. Cells were incubated with [4-^{14}C]testosterone, extracted, and subjected to TLC according to the method of Waxman et al. (1983).

Table I: Kinetic Parameters for Testosterone Metabolism by Wild-Type and Mutant CYP2A1

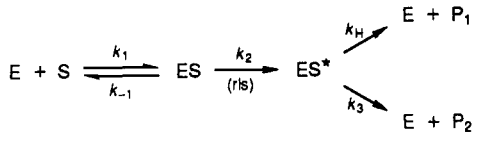
mutant		K_m (SD)[a]	V_{max} (SD)	V_{max}/K_m
wild type		0.32 (0.04)	20.50 (0.50)	64.06
2A1-1	N279Y	0.18	14.80	82.22
2A1-2	G280V	0.23	14.30	62.10
2A1-3	K287N	0.21	18.80	89.52
2A1-4	T292S	not active		
2A1-5	T293S	0.20	18.70	93.50
2A1-6	S295G	0.28	15.80	56.40
2A1-7	F297L	0.23	13.20	57.39
2A1-8	S301T	0.29	11.60	40.00
2A1-9	E302G	0.75	1.60	2.13
2A1-10	T303S	0.24 (0.06)	42.50 (2.50)	177.08
2A1-11	R309Y	0.75	2.60	3.47
2A1-12	Y310H	0.90	4.07	4.52
2A1-13	Q331R	not active		
2A1-7,10		0.19	11.00	57.89
2A1-9,10		0.80	3.00	3.75

[a] Averages and standard derivatives of five experiments are given for the wild type and 2A1–10 mutants. All other values are from single experiments for which $R^2 > 0.99$.

The kinetic data presented in Table I give the K_m and V_{max} values for the metabolism of testosterone by P450 2A1 and its mutants. The observed V_{max} value for the expressed wild-type enzyme (20.5 min^{-1}) was approximately 30% lower than was previously reported for the reconstituted enzyme (30.5 min^{-1}) while the K_m was 60% lower. The lower K_m was found to be due to the amount of the solvent methanol added to the incubations with the substrate. In the kinetic data reported here, a minimal amount of methanol (0.06%) was used in the reactions compared to the incubations in the previous study (2% methanol). The K_i for methanol was found to be ~500 mM (2%, data not shown).

The data in Table I show large differences in K_m, V_{max}, and V_{max}/K_m for substitutions in positions 9–12. Single point mutants 9, 11, and 12 and double mutant 9,10 have K_m values which are higher than wild type and V_{max} values which are lower. In contrast, mutant 10 has a normal K_m and a V_{max} value which is twice as high as wild type. The marked increase in K_m associated with mutants 9, 11, 12, and 9,10 most likely reflects poorer binding characteristics since their V_{max} values decrease.

Although the model in Scheme I has numerous rate constants involved in both the K_m and V_{max} values, isotope effect data can be used to estimate the amount of water formation. An analysis of the isotope effect kinetics for cytochrome P450 mediated oxidations suggests that the ability to observe an intermolecular isotope effect is due to the presence of multiple pathways (Korzekwa et al., 1989). In general, if the isotopically sensitive step is not rate limiting and if there is a prior irreversible step, an isotope effect will be masked, i.e., the observed effect will be less than the intrinsic isotope effect. However, if the isotopically sensitive pathway is in competition with a significant alternate pathway, switching to the alternative pathway can unmask an isotope effect. The following simplified scheme and equation describe this relationship:



$$(k_H/k_D)_{obs} = \frac{k_H/k_D + k_H/k_3}{1 + k_H/k_3} \quad (1)$$

where k_2 is a rate-limiting step, k_H is the isotopically sensitive step, and k_3 is the alternative pathway. As the k_3 increases, the observed isotope effect approaches the intrinsic value. Alternate pathways which can unmask P450 isotope effects include the formation of other oxidation products and the further reduction of the oxygenating species to water. In a previous study, isotope effects were measured for the metabolism of three deuterium-substituted testosterones with reconstituted P450 2A1. Although the study was primarily concerned with the mechanism of the formation of a minor metabolite, it was noted that formation of 7α-hydroxytestosterone was accompanied by a significant normal isotope effect ($\sim$3) when deuterium was incorporated in the 7α-position. Preliminary stoichiometry studies showed that the isotope effect is accompanied by an increase in the amount of water formation. This suggests that the isotope effect data for 7α-hydroxylation of testosterone can be used as an indicator for the amount of water formation. Unfortunately, the conditions used to perform the stoichiometry studies (100 mM potassium phosphate, pH 7.4, 30 °C) markedly affect the velocity of the reaction.[1] Therefore, while we observed switching to water formation, the observed stoichiometry cannot be used for analysis of the data presented here. Another difficulty with the quantitative analysis of the isotope effects data is that two of the deuterated substrates have 74% deuterium in the 6α- and 7α-positions with 26% in the 6β- and 7β-positions. As reported previously, the isotope effects can be corrected provided that any secondary isotope effects associated with the 7β-deuterio substrates are ignored.

Table II: Isotope Effects for 7α-Hydroxylation of Testosterone by Wild-Type and Mutant CYP2A1

mutant	k_H/k_D (sd)[a]	IE corr[b]
wild type	1.87 (0.03)	2.69
wild type (NADH)	1.37 (0.06)	1.57
2A1-1 N279Y	1.77 (0.05)	2.43
2A1-2 G280V	2.06 (0.08)	3.28
2A1-3 K287N	2.09 (0.07)	3.39
2A1-4 T292S	not active	
2A1-5 T293S	2.01 (0.12)	3.12
2A1-6 S295G	1.50 (0.05)	1.82
2A1-7 F297L	2.94 (0.05)	9.23
2A1-8 S301T	2.33 (0.04)	4.37
2A1-9 E302G	1.77 (0.02)	2.43
2A1-10 T303S	1.56 (0.03)	1.94
2A1-11 R309Y	2.36 (0.08)	4.52
2A1-12 Y310H	1.90 (0.01)	2.78
2A1-13 Q331R	not active	

[a] Average isotope effect from triplicate incubations; standard deviation after propagation of errors. [b] Isotope effects corrected for nonspecific incorporation as in Korzekwa et al. (1990).

The isotope effect data are given in Table II. Also included are the values corrected for lack of specific incorporation into the α-positions. It should be noted that, with the expected secondary isotope effects, the corrected isotope effects are most likely 15–30% too high.[2] An initial observation for the isotope effect data in Table II is that the observed effect for the expressed 2A1 is substantially lower than that for the reconstituted enzyme. This suggests that the active oxygenating form of the enzyme is more efficiently coupled to substrate oxidation for the expressed system. When NADH was used to support the reaction for the wild-type enzyme, the rate of oxidation of testosterone was decreased by 40% (data not shown), and the isotope effect decreased from 1.87 to 1.37. The lower isotope effect for the NADH-supported reaction suggests that substrate oxidation is in direct competition with reduction-dependent processes. This is what would be expected if water formation is initiated by the introduction of a third electron. Also, for this change in isotope effect to be observable, no irreversible steps can exist between the branch point for substrate oxidation and further reduction. Therefore, the isotope effect data provide information concerning the rate of reduction of the enzyme.

One may expect that the changes in V_{max} seen in Table I could be due to one or more factors. The lower values of V_{max} seen for mutants 9, 11, 12, and 9–10 could be due to (a) more water formation, (b) slower rate of reduction, (c) more hydrogen peroxide release, or (d) a slower overall rate caused by one or more decreased rate constants associated with oxygen activation or substrate oxidation. Contrarily, the higher V_{max} value associated with the 10 mutant may be due to (a) less water formation, (b) faster reduction, (c) less hydrogen peroxide release, or (d) a faster overall rate caused by one or more increased rate constants associated with oxygen activation or substrate oxidation. Some of these possibilities can be eliminated by considering the isotope effect data.

Since the isotope is directly related to the amount of water formed, the first possibility is the easiest to evaluate. The

[1] The turnover numbers of both the expressed and reconstituted system in 100 mM potassium phosphate buffer are approximately 50% of that in 50 mM phosphate buffer. The ratios of products (H_2O_2:metabolites:H_2O) were 1.0:0.64:0.31 for the protio substrate and 1.0:0.22:0.48 for the deuterio substrate.

[2] The equation for the corrected isotope effect which includes the α-secondary isotope effect for abstracting the 7α-hydrogen atom in the presence of a 7β-deuterium would be

$$V_H/V_D = (F_D V_{Dobs})/(1 - F_H V_{Dobs}/IE)$$

where F_H and F_D are the fractions hydrogen and deuterium in the 7α-position, V_{Dobs} is the observed isotope effect on V_{max}, and IE is the secondary isotope effect [see Korzekwa et al. (1990) for details].

substantially lower V_{max} values for mutants 9, 11, and 12 cannot be due to increased water formation since the isotope effects are not higher than that for the wild-type enzyme. However, the higher V_{max} value for the 10 mutant may be due partially to decreased water formation because the lower isotope effect implies less water formation.

As seen for the experiment using NADH instead of NADPH, the 40% decrease in testosterone hydroxylation rate was accompanied by a lower isotope effect, implying that less water is formed. The mutants with the lowest V_{max} values have isotope effects that are generally unchanged or higher than that of the wild-type enzyme. This suggests that the lower V_{max} value is not due to diminished ability for the enzyme to be reduced, unless the branching ratio is kept constant by a simultaneous decrease in the rate constant for hydrogen atom abstraction. Similarly, the higher V_{max} value for the 10 mutant should be accompanied by a higher isotope effect if the rate of reduction is increased, whereas a lower value is observed.

This leaves uncoupling to hydrogen peroxide (c) and other rate constants changes (d) as potential determinants for effects on V_{max}. If the overall electron flux remains constant at saturating substrate concentrations, then the amount of hydrogen peroxide formation is the likely determinant for changes in V_{max}. For mutant 10, the lower isotope effect suggests that less water is formed. However, the corrected value of the wild-type isotope effect suggests that the branching ratio (product/water) is greater than 1.0. For an intrinsic isotope effect of 10, the ratio would be 4.3 (eq 1), suggesting that the turnover number for water formation is 5 for the wild-type enzyme. The increase in V_{max} by 20 cannot be explained by water alone.

If the changes in V_{max} are due to changes in the rate of electron flux through the system, an increase in the steady-state concentration of at least one intermediate is required. It is unlikely that a decreased rate of hydrogen atom abstraction is responsible for the lower V_{max} values of the 9, 11, 12, and 9–10 mutants, since the amount of active oxygenating species is limited by water formation. Slowing of the hydrogen abstraction step would increase the branching ratio resulting in an increased isotope effect.

Presumably, the only other rate constants involved in oxygen activation would be those for proton transfers and that for heterolytic cleavage of the dioxygen bond. While the exact sequence of proton transfers associated with peroxide release and heterolytic cleavage are unknown, proton transfer could conceivably affect V_{max} in one of two ways. First, a mutation may result in an active site structure in which protons are not available. This would result in an increase in the intermediates prior to heterolytic cleavage. The second possibility is that a change in the proton environment of the active site promotes hydrogen peroxide formation. This has been postulated recently by Raag and Poulos (1991) to explain the uncoupling seen with certain substrates and P450cam. X-ray crystal structures were obtained for P450cam complexes with several substrates, two with metabolism reported to be inefficiently coupled to NADPH oxidation (Atkins & Sligar, 1989a,b). The authors suggest that the presence of one or more disordered water molecules in the active site during oxygen activation may be responsible for the increase in hydrogen peroxide and/or water formation. Evidence given to support this hypothesis is (1) that substrates that are uncoupled show high mobility in the active site, (2) some substrates are missing an ordered water molecule present in efficiently coupled systems while showing a positive difference density near the oxygen-binding site, and (3) the atomic temperature factors are higher for the amino acids in the dioxygen-binding groove for poorly coupled substrate complexes, suggesting the presence of disordered water.

Interestingly, on the basis of a previously reported sequence alignment of P450 2A1 with P450cam (Gotoh & Fujii-Kuriyama, 1989), the four mutants in this study with the low V_{max} values have substitutions within −1 to +7 amino acids from the conserved dioxygen-binding region of the I helix. Also, the low V_{max} values are all associated with higher K_m values. It may be expected that if water was present in the active site, the binding constant for a hydrophobic substrate such as testosterone would be decreased. The fact that the mutants tend to have either normal K_ms and V_{max} values or high K_m and low V_{max} values seems to support the hypothesis of Raag and Poulos that uncoupling can be associated with very few water molecules in the active site. If many water molecules are present in the active site and the amount of uncoupling and the binding constant is a function of the number, and the kinetic behavior of the mutants would be more varied rather than divided into two general groups. Also, if the higher V_{max} is due to the exclusion of many water molecules, a lower K_m might be expected to accompany the higher V_{max}.

Finally, if efficient metabolism requires that active sites be anhydrous in the presence of substrate, a rigid active site would result in high substrate specificity. Therefore, flexibility of the surrounding protein may be a common characteristic of the active sites for cytochrome P450s that have broad substrate and regioselectivity.

Conclusions

In conclusion, none of the single point mutants resulted in the loss of the specificity associated with the wild-type P450 2A1 and 2A2 enzymes. However, four of the single point mutants have changed kinetic parameters; three with higher K_m and lower V_{max} values and one with a V_{max} twice as high as that of wild type. The isotope effects for the metabolism of testosterone suggest that the changes in V_{max} are not primarily due to changes in reduction rates or changes in the amount of excess water formation. Therefore, it is likely that the V_{max} values are determined by the amount of hydrogen peroxide which is released. The fact that the mutations occur in the conserved oxygen-binding region, supports the hypothesis of Raag and Poulos (1991) that the release of hydrogen peroxide is due to the presence of disordered water molecules in the active site. Further studies are underway to determine directly the amount of hydrogen peroxide released as well as the complete stoichiometry of these reactions.

Registry No. Cytochrome P450, 9035-51-2; testosterone hydroxylase, 42616-24-0; testosterone, 58-22-0; deuterium, 7782-39-0.

References

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

Atkins, W. A., & Sligar, S. G. (1987) *J. Am. Chem. Soc. 109*, 3754–3760.

Atkins, W. A., & Sligar, S. G. (1989a) *J. Biol. Chem. 263*, 18842–18849.

Atkins, W. A., & Sligar, S. G. (1989b) *J. Am. Chem. Soc. 111*, 2715.

Gillette, J. R., Brodie, B. B., & La Du, B. N. (1957) *J. Pharmacol. Exp. Ther. 119*, 532–540.

Gonzalez, F. J. (1989) *Pharmacol. Rev. 40*, 243–288.

Gonzalez, F. J., Aoyama, T., & Gelboin, H. V. (1991) *Methods Enzymol. 206*, 85–92.

Gotoh, O., & Fuji-Kuriyama, Y. (1989) in *Frontiers in Biotransformation* (Ruckpaul, K., & Rein, H., Eds.) Vol. 1, pp 195-234, Akademie-Verlag, Berlin, and Taylor and Francis, London.
Gorsky, L. D., Koop, D. R., & Coon, M. J. (1982) *J. Biol. Chem. 259*, 6812-6817.
Guengerich, F. P., Ed. (1987) *Mammalian Cytochromes P-450*, Vols. 1 and 2, CRC Press, Roca Raton, FL.
Hanioka, N., Korzekwa, K., & Gonzalez, F. J. (1990) *Protein Eng. 3*, 571-575.
Iwasaki, M., Juvonen, R., Lindberg, R., & Negishi, M. (1991) *J. Biol. Chem. 266*, 3380-3382.
Korzekwa, K. R., Trager, W. F., & Gillette, J. R. (1989) *Biochemistry 28*, 9012-9018.
Korzekwa, K. R., Trager, W. F., Nagata, K., Parkinson, A., & Gillette, J. R. (1990) *Drug Metab. Dispos. 18*, 974-979.
Kronbach, T., Larabee, T. M., & Johnson, E. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8262-8265.
Lindberg, R. L. P., & Negishi, M. (1989) *Nature 339*, 632-634.
Lorence, M. C., Trant, J. M., Clark, B. J., Khyatt, B., Mason, J. I., Estabrook, R. W., & Waterman, M. R. (1990) *Biochemistry 29*, 9819-9824.
Morgan, E. T., Koop, D. R., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 13591-13597.
Nebert, D. W., Nelson, D. R., Coon, M. J., Estabrook, R. W., Feyereisen, R., Fuji-Kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R., & Waxman, D. J. (1991) *DNA 10*, 1-14.
Nelson, D. R., & Strobel, H. W. (1989) *Biochemistry 28*, 656-660.
Nordblom, G. D., & Coon, M. J. (1982) *Arch. Biochem. Biophys. 180*, 343-347.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2379-2385.
Ortiz de Montellano, P. R., Ed. (1986) *Cytochrome P-450, Structure, Mechanism and Biochemistry*, Plenum Press, New York.
Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195*, 687-700.
Prevelige, P., Jr., & Fasman, G. (1989) in *Prediction of Protein Structure and Principles of Protein Conformation* (Fasman, G., Ed.) pp 391-416, Plenum Press, New York.
Raag, R., & Poulos, T. L. (1991) *Biochemistry 30*, 2674-2684.
Shimizu, T., Hirano, K., Takahashi, M., Hatano, M., & Fuji-Kuriyama, Y. (1988) *Biochemistry 27*, 4138-4141.
Shimizu, T., Sadeque, A. J. M., Sadeque, G. N., Hatano, M., & Fuji-Kuriyama, Y. (1989) *Biochemistry 30*, 1490-1496.
Shimizu, T., Tateishi, T., Hatano, M., & Fuji-Kuriyama, Y. (1991) *J. Biol. Chem. 266*, 3372-3375.
Uno, T., & Imai, Y. (1989) *J. Biochem. (Tokyo) 106*, 569-574.
Waxman, D. J., Ko, A., & Walsh, C. (1983) *J. Biol. Chem. 258*, 11937-11947.

Direct Relationship between Intracellular Calcium Mobilization and Phospholipase D Activation in Prostaglandin E-Stimulated Human Erythroleukemia Cells†

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ABSTRACT: The relationship between calcium mobilization and phospholipase D (PLD) activation in response to E-series prostaglandins (PGEs) was investigated in human erythroleukemia cells. Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was increased by PGE_1 and PGE_2 over the same concentration range at which PLD activation was seen. Pretreatment of cells with pertussis toxin greatly inhibited the PGE-stimulated increase in $[Ca^{2+}]_i$, implying that a G protein participates in the PGE receptor signaling process. The peak level and also the plateau level of Ca^{2+} mobilization stimulated by these prostaglandins were markedly decreased in Ca^{2+}-depleted medium, indicating that both extracellular and intracellular Ca^{2+} stores contribute to the changes in $[Ca^{2+}]_i$. Likewise, activation of PLD by PGE_1 and PGE_2 was abolished by pertussis toxin pretreatment or incubation in Ca^{2+}-depleted medium. U73122, a putative phospholipase C inhibitor, blocked both Ca^{2+} mobilization and PLD activation in PGE-stimulated cells. Furthermore, the intracellular loading of BAPTA, a Ca^{2+} chelator, inhibited both Ca^{2+} mobilization and PLD activation by PGE_1 and PGE_2 in a similar dose-dependent manner. Simultaneous measurement of $[Ca^{2+}]_i$ and PLD activity in the same cell samples indicated that PLD activity increases as a function of $[Ca^{2+}]_i$ in a similar fashion in cells stimulated either by PGEs or by the calcium ionophore ionomycin. Taken together, these findings suggest that a rise in $[Ca^{2+}]_i$ is necessary for PGE-stimulated PLD activity in human erythroleukemia cells.

Hydrolysis of phospholipids by phospholipase D (PLD)[1] yields phosphatidic acid (PA) and polar head group "bases" such as choline and ethanolamine (Heller, 1978). PA may itself act as a second messenger to elicit cellular effects (Ohsako & Deguchi, 1981; Moolenaar et al., 1986; Murayama & Ui, 1987) or be further metabolized by PA phosphohydrolase to diacylglycerol (Billah et al., 1989), an activator

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[1] Abbreviations: HEL cells, human erythroleukemia cells; IP_3, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PEt, phosphatidylethanol; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D; PMA, 4β-phorbol 12-myristate 13-acetate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; MTH medium, modified Tyrode-Hepes medium.

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