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CHIMERIC cDNA EXPRESSION AND SITE DIRECTED MUTAGENESIS STUDIES OF CYTOCHROME *P*450s CYP2A1 and CYP2A2

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Summary—Construction of chimeras and site directed mutagenesis were used to study the regioselectivity and kinetics of testosterone hydroxylation by the cytochrome *P*450s CYP2A1 and CYP2A2. Although these enzymes exhibit 88% sequence similarity, they catalyze very different regioselective hydroxylations of testosterone. Active chimeras in which the first 355 amino acids do not correspond to a single enzyme show broad radioselectivity, whereas the specificity of the parent enzyme is obtained if the first 355 amino acids are unchanged. Therefore, the region between amino acids 275 and 355 is important in maintaining regioselectivity. Single point mutants were constructed for the 13 amino acid differences in this region. For 26 single point and 2 double mutants all active mutants have the same regioselectivity as the parent enzymes. However, kinetic analysis of the CYP2A1 mutants showed that 4 single point mutants and 1 double mutant had kinetic parameters very different from the parent enzyme. All of these substitutions are associated with the conserved dioxygen binding region of the putative I helix predicted from the crystal structure of $P450_{cam}$. Deuterium isotope effects were used to determine any changes in the rate of reduction and to estimate the relative amount of excess water formation. Changes in reduction rates are not sufficient to account for the differences in V_{max} values. Therefore, it is likely that the amount of hydrogen peroxide formed is a primary determinant of V_{max}.

INTRODUCTION

The cytochrome *P*450s are a superfamily of monooxygenases involved in the metabolism of both exogenous and endogenous compounds. Due to their primary importance in drug metabolism and steroidogenesis, these enzymes have been extensively studied for the last 25–30 years [1]. Although there has been a tremendous effort to determine the details of the catalytic cycle, several aspects of these enzyms have hindered their complete characterization. These include the absence of crystal structures (for mammalian enzymes) the complexity of the catalytic cycle, presence of multiple isozymes, the diversity of substrates (for some isozymes), and the versatility of the active oxygen species. While many of the mechanisms of the actual substrate oxidation steps have been defined, the aspects of these enzymes responsible for substrate specificity and catalytic efficiency are still unknown.

The cDNA cloning and expression of several of the cytochrome *P*450s has provided many of the recent advances in this field. While the comparison of *P*450 primary amino acid sequences shows extreme divergence between the different families of enzymes, heme and oxygen binding regions have been highly conserved for all cytochrome *P*450s. Also, secondary structure predictions suggest that many of the structural elements of the cytochrome *P*450s have been conserved. This has allowed for alignments to be made with $P450_{cam}$ [2, 3] the bacterial enzyme for which a high resolution crystal structure is available [4]. Chimera construction and site directed mutagenesis have provided information on many aspects of several *P*450s [5–14]. Recently, a sequence alignment of many cytochrome *P*450 family 2 proteins [15] has shown that all mutagenesis data can be explained by substrate binding regions that align with those for $P450_{cam}$, including the substrate binding region for the mouse 2A enzymes described by Negishi and coworkers [7–9].

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CYP2A1 AND CYP2A2

The cytochrome *P*450s CYP2A1 and CYP2A2 are rat liver enzymes showing differential expression. CYP2A1 is repressed in adult males and active in females whereas CYP2A2 is an adult male specific enzyme [16]. Although these enzymes exhibit 88% amino acid similarity, their catalytic activities are quite different. CYP2A1 metabolizes testosterone specifically in the 7α-position (90 with 10% 6α metabolism) [17] and CYP2A2 metabolizes testosterone in several positions with 15α- and 12α-hydroxytestosterone being the predominant products [16, 18].

Chimera analysis

Initial experiments in our laboratory involved the construction of chimeras between CYP2A1 and CYP2A2 [19]. Four chimera pairs were constructed based on the restriction sites shown in Fig. 1. These chimeric cDNAs were inserted into the vaccinia virus expression system and the chimeric *P*450 proteins were expressed in Hep G2 cells [20]. Chimera Y1 is a construct with the first 162 amino acids corresponding to CYP2A1 and the last 330 to CYP2A2 whereas Y2 is the diametric opposite. For the S and X chimeras, the fragments were extended to 275 and 356 amino acids, respectively. The last pair of chimeras are constructed from the *Ban* II restriction sites at positions 162 and 447, with B1 corresponding to CYP2A1 for the first 162 and the last 45 amino acids and the opposite for chimera B2.

The testosterone hydroxylase activities and product profiles were measured and are shown in Fig. 1. Chimeras S1 and B1 had no measurable testosterone hydroxylase activity. This may be due to the instability of the protein, as Western immunoblotting shows a much lower level of protein. Chimeras S2 and B2 have 7α-, 6α- and 12α-hydroxylase activities in relative proportions similar to CYP2A2, but are missing the major metabolite, 15α-hydroxytestosterone. For chimeras X1 and X2 where the first 356 amino acids duplicate CYP2A1 and CYP2A2, respectively, the product profiles match their respective parent enzymes. Interestingly, for chimeras Y1 and Y2, in which only the first 162 amino acids have been changed, the same metabolite profiles are obtained for both chimeras. Again this profile is that of CYP2A2 less 15α-hydroxytestosterone.

These results can be interpreted as follows. First, product profiles are determined by the *N*-terminus with a length between 275 and 356 amino acids for CYP2A2 and between 162 and 376 amino acids for CYP2A1. Second, it appears that both specific interactions and

enzyme/chimera	Testosterone Metabolites (pmol/min,mg prot.) 7α	6α	6β	15α	12α
CYP2A1	338	14	---	---	---
CYP2A2	19	---	19	88	61
B1	inactive				
B2	16	---	22	---	40
S1	inactive				
S2	2	---	2	---	6
X1	23	2	---	---	---
X2	8	---	10	36	36
Y1	3	---	4	---	7
Y2	16	---	22	---	40

Fig. 1. Structure and regioselectivity of CYP2A1 and CYP2A2 chimeras. For experimental details see Ref. [19].

non-specific interactions are involved in the binding of testosterone to CYP2A2. For example, there is a binding interaction favorable for 15α-hydroxylation which is present with the chimeric protein having 356 CYP2A2 amino acids which is not present in proteins of shorter CYP2A2 contributions. This could be due to either a favorable binding contribution for chimera X2 or an unfavorable interaction, i.e. steric hinderance, for the S2, Y2, and B2 proteins. Both possibilities could be due to either specific contributions by the region between amino acids 275 and 356 or significant conformational changes when this region is substituted. A similar situation exists for the CYP2A1 chimeras. Again, 356 amino acids are required to manifest CYP2A1 specificity. The most unusual result is that Y1 and Y2 have the same broad regioselectivity which is similar to the B2 and S2 chimeras. From this data, it is tempting to speculate that the active sites for both CYP2A1 and CYP2A2 are similar with non-specific binding interactions that result in 7α, 6β- and 12α-hydroxylase activities. Specific binding interactions would then result in 15α-hydroxylation by CYP2A2 and the high specificity for hydroxylation at the 7α-position by CYP2A1.

Single point mutants of CYP2A1 and CYP2A2

Based on the above analysis, specific binding interactions, at least for CYP2A2, would be expected to be due to the amino acids between 276 and 356. Sequence alignments with $P450_{cam}$ [3] suggests that this region corresponds to the I and J helices. The I helix (distal helix) of $P450_{cam}$ passes over the heme and is a part of the substrate and oxygen binding sites. Since there are 13 amino acid differences in this region between CYP2A1 and CYP2A2 (Fig. 2). these were changed individually and inserted into the vaccinia virus vector for expression in Hep G2 cells [21]. Preliminary analysis showed that of 26 single point and 2 double mutants, 3 were inactive and the others had the same regioselectivity as their parental counterparts. Three of the 4 inactive mutants show a low level of protein, suggesting that they are unstable and subject to degradation. Since no single amino which has been changed (and resulted in a stable enzyme) is entirely responsible for the regioselectivities of CYP2A1 and CYP2A2, it is likely that conformational changes are involved.

Initial screening by *in situ* analysis of testosterone metabolism suggested that changes in rates of hydroxylation are observed with some of the mutants. Since the kinetic parameters and mechanistic aspects of CYP2A1 have been studied previously [17], Lineweaver–Burke plots were generated for all CYP2A1 mutants (Fig. 3). This data shows that the mutants fall into two general groups: those that have normal K_m and V_{max} values (1–8) and those that have

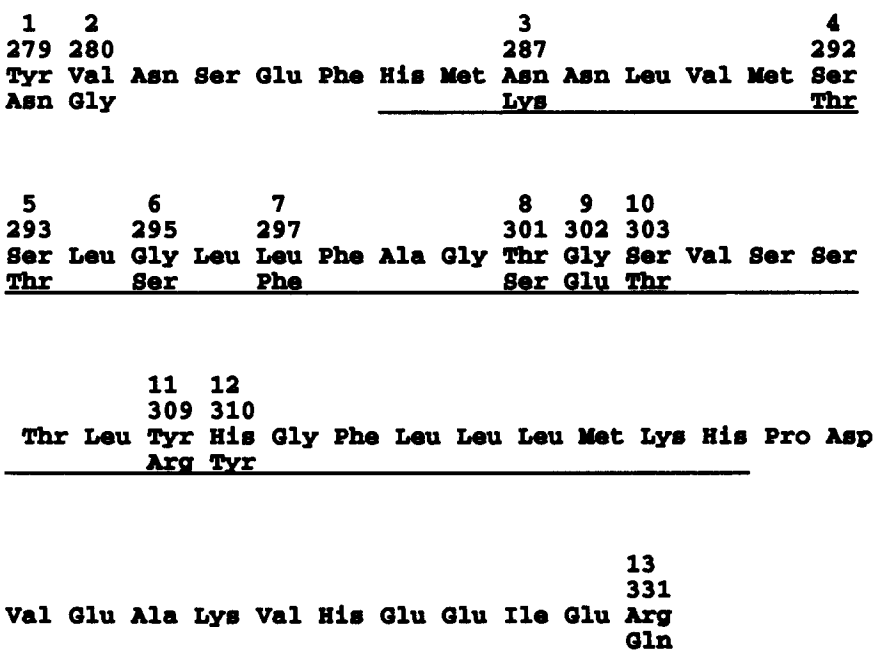


Fig. 2. Region of the 2A *P*450s in which the mutations occur. The sequence given is for *P*450 2A2 and 2A1 substitutions are shown below. The underlined sequence is the region which aligns with the I helix of *P*450cam.

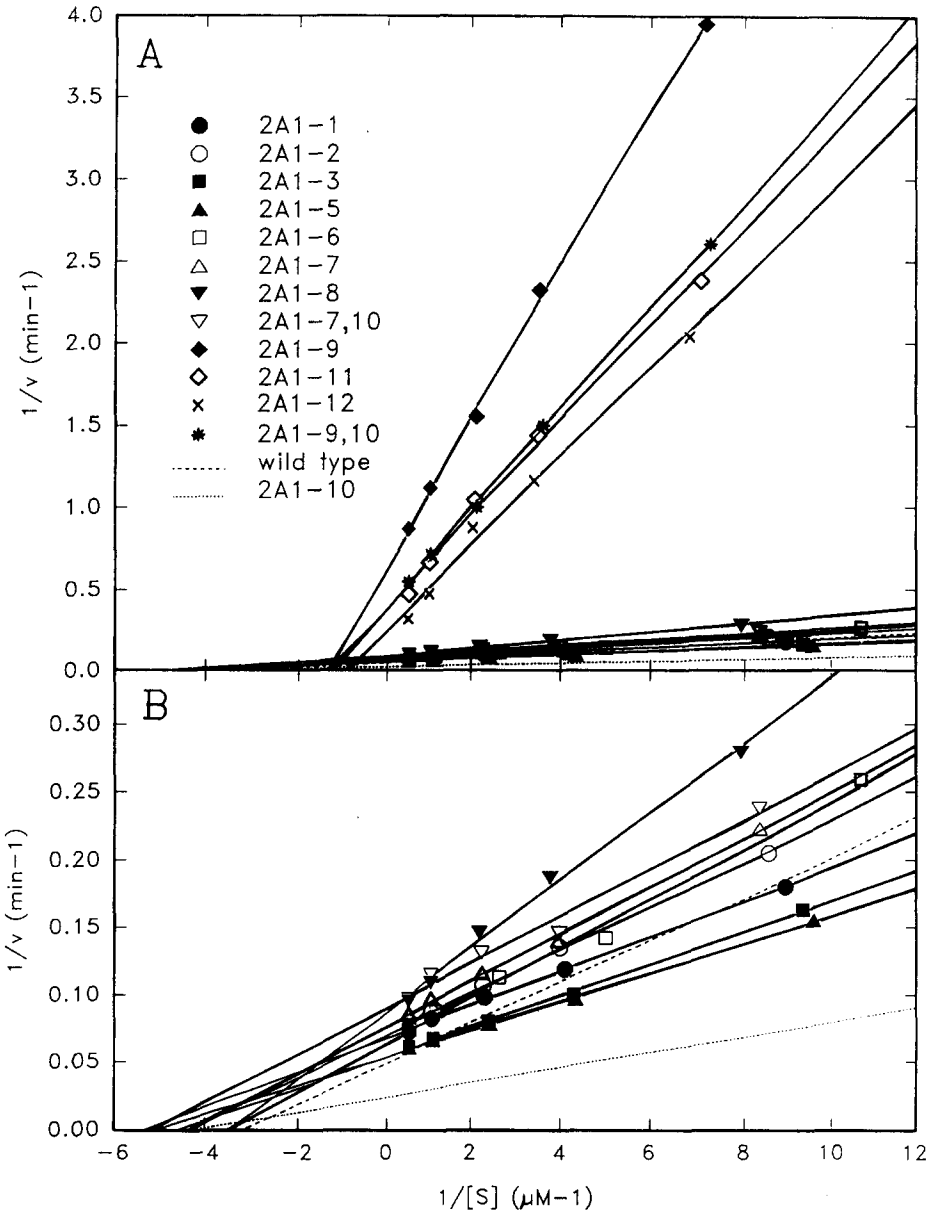
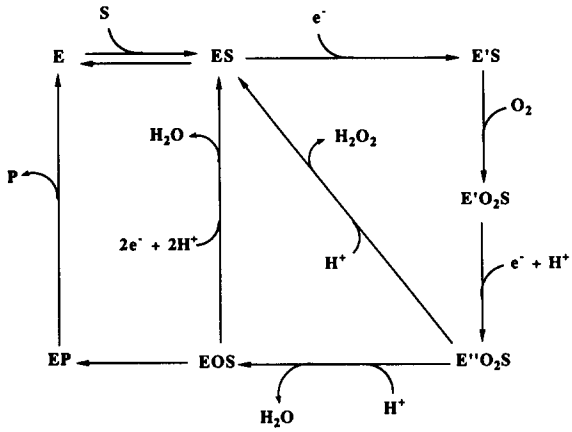


Fig. 3. Double reciprocal plots of wild-type *P*450 2A1 and mutants. Plot (B) includes the high V_{max} mutants plotted on an expanded scale. The lines corresponding to wild-type and the 2A1-10 mutant are averages of five determinations. Enzyme concentrations were measured by CO reduced spectral analysis and metabolism was quantitated by HPLC. For experimental details see Ref. [21].

high K_m and low V_{max} values (9, 11, 12). In addition, mutant 10 has a normal K_m and a V_{max} that is twice as high as the wild-type enzyme. Thus, mutants 9–12 show kinetic characteristics which are significantly different from CYP2A1. This region aligns with the portion I helix which is part of the active site of $P450_{cam}$. Interestingly, mutant 10 (Thr-303-Ser) aligns with Thr-252 of $P450_{cam}$, the residue which is thought to directly bind molecular oxygen, suggesting that the alignments of cytochrome *P*450s across the conserved oxygen binding regions may be appropriate, at least for the I helix.

The analysis of the kinetics of any cytochrome *P*450 is complicated by the multi-step nature of catalytic cycle. In addition to the normal steps associated with oxygen activation (oxygen binding, reductions, heterolytic cleavage, etc.) two decoupling pathways have also been shown to exist [22]. These are the release of hydrogen peroxide before heterolytic cleavage and the further reduction of the active oxygenating species after heterolytic cleavage.



Scheme 1

Although it is difficult to directly quantitate the amount of hydrogen peroxide formed, particularly for cell preparations, the relative amount of excess water formation can be estimated by isotope effect experiments.

It is becoming apparent that because of the complexity, irreversibility and long time scale of the *P*450 catalytic cycle (Scheme 1), manifestation of an intermolecular isotope effect for a *P*450 mediated hydrogen abstraction reaction requires that an alternate pathway be in competition with the isotopically sensitive step [23]. This pathway can be either an alternate position of metabolism or the introduction of another electron to initiate water formation. At steady-state, without the presence of an alternate pathway, substitution of deuterium in a substrate will result in an increase in the active-enzyme/substrate complex and mask the observed isotope effect. The alternate pathway simply prevents the buildup of this complex and unmasks the observed isotope effect. Previous studies in our laboratory have shown that for CYP2A1, water formation is in direct competition with hydrogen abstraction from the 7α-position [17, 21]. Direct evidence was provided by observing an increase in water formation when dueterium was present in the 7α-position. Furthermore, the use of NADH instead of NADPH as the source of reducing equivalents resulted in a large decrease in both V_{max} and the observed isotope effect. Since the degree of unmasking of an isotope effect by an alternate pathway increases as the branching ratio to the alternate pathway increases, the lower isotope effect associated with slower reduction rates suggests that the rate of reduction of the active oxygen species determines the rate of water formation. Conversely, any changes in the isotope effect are likely to be due to changes in the branching ratio, provided that the mechanism of oxidation does not change.

The above analysis was used to interpret the isotope effects observed for the wild-type and mutant CYP2A1 enzymes (Table 1). For the mutants with substantially lower V_{max} values, the isotope effects were approximately the same as the wild-type enzyme. This suggests that the lower V_{max} value was not due to an increase in water formation. Also, since the rate of water formation is dependent on the rate of reduction, this data suggests that the overall rate of reduction of the enzyme has not been changed by the mutations which result in lower V_{max} values. The 2A1-10 mutant (T303S) has a V_{max} value which is twice as high as the wild-type enzyme. The lower isotope effect suggests that some of the increase in V_{max} may be due to a decrease in water formation. It also suggests that this increase is not a result of an increase in the rate of reduction since this would increase the isotope effect. However, based on the observed change in the isotope effect, the decrease in the amount of water formed is too small to account for the observed increase in V_{max}. Therefore, the isotope effect data for mutants 2A1-9 to 2A1-12 suggests that another factor is the primary determinant of V_{max} for this enzyme.

The most likely candidate for this determinant is the amount of hydrogen formation. For the wild-type enzyme (at 100 mM Kpi, 30°C) approx. 50% of the reducing equivalents

Table 1. Isotope effects for 7α-hydroxylation of testosterone by wild-type and mutant CYP2A1

Mutant		k_H/k_D (sdev)[a]	Mutant		k_H/k_D (sdev)[a]
Wild-type		1.87 (0.03)	2A1-7	F297	2.94 (0.05)
Wild-type	(NADH)	1.37 (0.06)	2A1-8	S301T	2.33 (0.04)
2A1-1	N279Y	1.77 (0.05)	2A1-9	E302G	1.77 (0.02)
2A1-2	G280V	2.06 (0.08)	2A1-10	T303S	1.56 (0.03)
2A1-3	K287N	2.09 (0.07)	2A1-11	R309Y	2.36 (0.08)
2A1-4	T292S	Not active	2A1-12	Y310H	1.90 (0.01)
2A1-5	T293S	2.01 (0.12)	2A1-13	Q331R	Not active
2A1-6	S295G	1.50 (0.05)			

[a] Average isotope effect from triplicate incubations. Standard deviation after propagation of errors.

result in hydrogen peroxide formation. Raag and Poulos [24] have recently suggested that uncoupling to hydrogen peroxide observed with the oxidation of certain substrates by $P450_{cam}$ may be associated with the presence of water molecules in the active site. This would suggest that the ability to efficiently metabolize a substrate requires the exclusion of water molecules from the active sight upon substrate binding. This hypothesis provides a possible explanation for the effects on the kinetics of testosterone hydroxylation by CYP2A1 and its mutants. The four mutations which alter the turnover number of the enzyme occur in the oxygen binding region of the I helix. If these mutations change the microenvironment of the active site, it may be expected that the number or orientation of water molecules present in the enzyme–substrate complex may be altered. An increase in the number of waters or an unfavorable change in their orientation may result in an increase in hydrogen peroxide formation, whereas a more anhydrous active site may result in a decrease in the amount of hydrogen peroxide. Since a large amount of hydrogen peroxide is formed for the wild-type enzyme, it might be expected that this amount may be altered by mutations in the oxygen binding region.

Implications for other P450s

The analysis of both the chimera data and the single point mutant data suggests that the active sites of CYP2A1 and CYP2A2 may have a certain amount of flexibility in their structure. Most of the active chimeras, even diametric opposites, have similar non-specific regioselectivities suggesting that non-specific binding (i.e. hydrophobic binding) is involved. Also, no single amino acid has been identified which is responsible for the specific binding interaction which results in 15α-hydroxylation for CYP2A2, suggesting that conformational changes are also involved in determining the specificities of the chimeras. If exclusion of water from the active site is necessary for the efficient metabolism of a substrate, a certain amount of conformational flexibility may be necessary for an enzyme to exclude water while binding a variety of substrates. Cytochrome $P450_{cam}$ and other *P*450s specific for one substrate may have rigid active sites, whereas *P*450s which have evolved for the metabolism of xenobiotics may have much "softer" active sites. This could explain why families of isozymes have evolved with specificities toward a general shape, i.e. CYP2A1 for polycyclic aromatic hydrocarbons, or electronic characteristics, i.e. CYP2A2 for protonated amines. This would require the apoproteins to conform to a general shape for efficient metabolism to occur.

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