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# Use of Chimeric Enzymes and Site-Directed Mutagenesis for Identification of Three Key Residues Responsible for Differences in Steroid Hydroxylation between Canine Cytochromes P-450 3A12 and 3A26

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## ABSTRACT

Canine cytochromes P-450 3A12 and 3A26 differ by 22 out of 503 amino acid residues. Chimeric constructs and site-directed mutants were used to identify the residues responsible for the much higher rates of steroid hydroxylation by 3A12. Six initial 3A12/3A26 hybrids were generated using convenient restriction sites, and site-directed mutagenesis was used to restore full 3A12 activity to two of the hybrids. One pair of 3A12/3A26 chimeras indicated that the first four residue differences between 3A12 and 3A26 were at least partially responsible for the differences in progesterone hydroxylation. Conversion in one of the hybrids of the Ile-187 residue found in 3A26 to the Thr in 3A12 conferred 3A12 levels of progesterone 6$\beta$-hydroxylase activity. Analysis of another chimera identified key residues within an internal *Pst*I fragment (codons 331–459) containing six amino acid residue differences. Subsequent site-directed mutagenesis of 3A26 residues Ser-368 and Val-369 to Pro and Ile, respectively, restored the rate of formation of 6$\beta$-hydroxyprogesterone by the hybrid to that of 3A12. The simultaneous conversion of 3A26 residues 187, 368, and 369 to those of 3A12 conferred greater than a third of the progesterone 6$\beta$-hydroxylase activity and all of the testosterone and androstenedione 6$\beta$-hydroxylase activity of 3A12. Addition of the carboxyl terminal 44 3A12 residues to the 3A26 triple mutant doubled progesterone 6$\beta$-hydroxylase activity. This is the first study to use catalytically distinct cytochromes P-450 3A from the same species in the elucidation of structure-function relationships.

Members of the cytochrome P-450 (P-450) superfamily of hemoproteins are responsible for the metabolism of a wide range of endogenous and exogenous compounds. The 3A enzymes are major contributors to hepatic biotransformation pathways, with human 3A4 accounting for as much as 60% of the P-450 found in human liver (Guengerich, 1995). A large number of clinically relevant drugs are metabolized by 3A enzymes, including cyclosporine, erythromycin, lidocaine, nifedipine, and steroids, as reviewed recently (Guengerich, 1995). In addition, numerous adverse pharmacokinetic drug interactions have been observed clinically with the concomitant use of multiple drugs that are metabolized by 3A enzymes (Periti et al., 1992). Despite the wealth of information on the importance, regulation, and substrate specificity of the P-450 3A subfamily, until recently relatively little was known about the structure-function relationships of these enzymes (Harlow and Halpert, 1997; He et al., 1997; Domanski et al., 1998). In contrast to the 2A (Lindberg and Negishi, 1989; Honkakoski and Negishi, 1997), 2B (Aoyama et al., 1989; Kedzie et al., 1991), and 2C (Kronbach et al., 1989; Hsu et al., 1993) subfamilies, a lack of functionally distinct natural variants and the high conservation of specificities across species has hindered structure-function analyses of the P-450 3A.

Canine models have been used extensively in drug metabolism studies, but much remains to be learned about the individual P-450 forms. Previous studies have demonstrated that canine P-450 3A12 catalyzes the hydroxylation of steroids including progesterone, testosterone, and androstenedione at rates comparable with human P-450 3A4 (Born et al., 1996; Fraser et al., 1997). In contrast, the 6$\beta$-hydroxylase

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**ABBREVIATIONS:** P-450, cytochrome P-450; PCR, polymerase chain reaction; androstenedione, androst-4-ene-3,17-dione; IPTG, isopropyl-$\beta$-D-thiogalactopyranoside; ALA, $\delta$-aminolevulinic acid; CHAPS, 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate; SRS, substrate recognition site.

activity of 3A26 with the same substrates was much lower, despite the fact that these two enzymes exhibit 96% amino acid sequence identity (Fraser et al., 1997). The relative rates of hydroxysteroid product formation were dependent upon the substrate and metabolite, with 3A26 displaying only 2% of the activity of 3A12 for 6$\beta$-hydroxyprogesterone formation but as much as 22% of the 3A12 activity for 2$\beta$-hydroxytestosterone (Fraser et al., 1997). These results indicated that canine P-450 3A12 and 3A26 might provide an excellent model system for the investigation of the structural basis of 3A substrate specificity.

Studies of P-450 2C enzymes in several laboratories have employed hybrid and hybrid/mutant constructs to identify amino acid residues critical for functional differences between P-450 2C4 and 2C5 (Kronbach et al., 1989), 2C2 and 2C14 (Uno and Imai, 1992), 2C3 and 2C3v (Hsu et al., 1993), 2C1 and 2C2 (Ramarao et al., 1995; Ramarao and Kemper, 1995), and 2C9 and 2C19 (Ibeanu et al., 1996). Prompted by these studies, the general strategy for the current investigation involved the use of hybrid enzymes in conjunction with site-directed mutants to identify the specific residue differences between 3A12 and 3A26 that account for their differences in steroid hydroxylation. Chimeric enzymes were generated with the goal of identifying limited regions of 3A12, replacement of which by 3A26 residues caused a significant loss of steroid hydroxylase activity. Back-mutation of individual amino acid residues was used to restore activity to the hybrids. The information from these chimeric mutants was employed to generate a 3A26 mutant with steroid hydroxylase rates similar to those of 3A12.

Progesterone was chosen as the substrate for the initial experiments employing chimeras and chimeric mutants, based on the extreme differences in the ability of 3A12 and 3A26 to catalyze hydroxylation of this steroid. Testosterone and androstenedione were then used in addition to progesterone in the analyses of mutant 3A26 constructs. Our findings indicate that residues at positions 187, 368, and 369 are instrumental in conferring differences in steroid hydroxylation rates observed between canine P-450 3A12 and 3A26.

## Experimental Procedures

**Materials.** Restriction endonucleases and media for bacterial growth were purchased from GIBCO-BRL (Grand Island, NY). The pSE380 expression vector used in all of these studies was purchased from Pharmacia (Alameda, CA). Primers for polymerase chain reaction (PCR) amplification and mutagenesis were obtained from Genosys, Inc. (Woodlands, TX). 3-((3-Cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS), progesterone, testosterone, androstenedione, NADPH, and dioleoylphosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). [4-$^{14}$C]Testosterone was obtained from Amersham Life Sciences (Arlington Heights, IL). [4-$^{14}$C]Progesterone and [4-$^{14}$C]androstenedione were obtained from Dupont-New England Nuclear (Boston, MA). HEPES was purchased from Calbiochem Corp. (LaJolla, CA). Thin-layer chromatography plates [silica gel, 250 $\mu$m, Si 250 PA (19C)] were purchased from J. T. Baker (Phillipsburg, NJ). All other reagents and supplies not listed were obtained from standard sources.

**Cloning and Expression of 3A12, 3A26, Hybrids, and Site-Directed Mutants.** The P-450 3A12 and 3A26 cDNAs were isolated from a $\lambda$gt11 cDNA library generated from canine liver as described previously (Ciaccio et al., 1991; Fraser et al., 1997). The N-termini of 3A12 and 3A26 are identical in sequence until the first variation is encountered at amino acid 111. Modifications to the N-terminus of 3A12 have been described previously (Born et al., 1996; Fraser et al., 1997). Restriction endonucleases and subcloning were used in the modification of 3A26 for expression in *E. coli*. The coding sequence for the unmodified N-terminus of 3A26 was removed and replaced with the corresponding segment that encodes the modified N-terminus of 3A12. These alterations removed ten amino acids in the signal anchor sequence of 3A26 and changed the second amino acid residue from aspartic acid to alanine, changes that have been shown to facilitate expression in *Escherichia coli* (Barnes et al., 1991; Gillam et al., 1993). 3A12 and 3A26 constructs and all chimeras and site-directed mutants were maintained in the pSE380 expression vector.

Heterologous protein expression and preparation of solubilized *E. coli* membranes was done essentially as described previously (John et al., 1994; Born et al., 1996; Fraser et al., 1997). All constructs were maintained in DH5$\alpha$ cells and grown at 37°C with 240 rpm shaking in 250 ml of liquid TB media (12 g Bacto tryptone, 24 g Bacto yeast extract, 4 ml glycerol/liter) to mid log phase. Isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) (final concentration 1.0 mM) and 80 mg/L $\delta$-aminolevulinic acid (ALA) were added, and cells were harvested after incubation at 30°C with 190 rpm shaking. Optimal expression of 3A26 was observed at 38 to 42 h after IPTG/ALA addition, and typical recovery of 3A26 protein ranged from 6 to 10 nmol/L of culture. Maximal expression of 3A12 was observed at 72 h after IPTG/ALA addition and yields ranging from 40 to 60 nmol/L of culture were routine. All chimeras and site-directed mutants were incubated for 38 to 42 h at 30°C after addition of IPTG/ALA to ensure adequate protein recovery based on the results obtained for 3A26.

**Generation of Chimeras and Site-Directed Mutants.** Chimeric combinations of 3A12 and 3A26 were generated using internal restriction sites by standard subcloning techniques. Plasmids containing 3A12 and 3A26 were cut with the appropriate enzymes and DNA fragments were separated on 1.0% agarose gels. The desired DNA fragments were purified using the GeneCleanII DNA purification kit. Chimeras of 3A12 and 3A26 were then generated by combining these fragments and religating them into complete constructs (Fig. 2). The *Dra*III site at bp 798 was used in conjunction with the *Hin*dIII site in the multiple cloning site (MCS) of pSE380 to separate the first 4 residue differences from the remaining 18. Similarly, the *PpuMI* site at bp 1371 was used with the *Hin*dIII site in the MCS to separate the first 14 residue differences from the last 8 changes. In addition to these modifications, an internal 450-bp *Pst*I fragment was exchanged between the constructs to separate the 6 variations found within this region from the 16 flanking differences (Fig. 2). The multiple hybrids and hybrid/mutants were generated by exchanging restriction fragments among single hybrid and mutant constructs. The *Pst*I fragment from 3A26 with the Pro-368 and Ile-369 from 3A12 was inserted into chimera A to generate the mutant/hybrid G in Fig. 4. Similarly, the *Pst*I fragment from 3A12 was inserted into the chimera A construct to make hybrid H and into chimera D to make hybrid J. In a similar fashion, the *Pst*I fragment from 3A26 was inserted into chimera C to generate hybrid K. Finally, hybrid/mutant L was made by inserting the *PpuMI* tail of 3A12 containing the last eight residue differences into the I187T/S368P/V369I 3A26 triple mutant construct.

Site-directed mutagenesis was accomplished using two different methods. The mutations at residues 368 and 369 were done using overlap PCR. Primers for modification of either or both of these residues were designed in the forward and reverse orientations and used with corresponding external primers to generate two fragments that overlap in the region containing the mutation. All mutagenic forward and reverse overlapping primers, with incorporated modifications underlined, are presented in Fig. 1. The external primers were designed to overlap the external *Dra*III and *Hin*dIII restriction sites at bp 798 and in the MCS, respectively. Reaction conditions were: 1 cycle of 94°C for 5 min followed by 29 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The resulting two PCR products were then used as template in conjunction with the external non-mutagenic primers in a second PCR reaction to generate a single

full-length fragment. This fragment was then isolated from an agarose gel and digested with the restriction endonuclease *Pst*I. The 450-bp fragment was then cloned into the appropriate vector fragments digested with *Pst*I, and positive clones were then checked for orientation and sequenced.

The I129 M, I187T, N209K, and L220F mutants were generated using overlapping forward and reverse primers (Fig. 1) containing the desired residue changes in conjunction with the high-fidelity *Pwo* polymerase (Boehringer Mannheim, Indianapolis, IN), which displays corrective exonuclease activity. The PCR reaction consisted of 1 cycle of 94°C for 5 min followed by 10 cycles of 94°C for 1 min, 60°C for 1 min, 68°C for 4 min, and a final 5-min extension at 68°C. This protocol resulted in the generation of full-length constructs with changes at the desired residues. The PCR products were then digested with *Dpn*I, which cuts only DNA strands that are methylated, thus removing the template plasmid from the reactions. The PCR reactions were directly transformed into DH5$\alpha$ competent cells, and DNA was isolated and analyzed for the desired alterations. DNA dideoxy-sequencing was performed on all constructs to ensure that no additional changes were incorporated into the constructs as a result of the PCR reactions. Positive mutants were then expressed and solubilized membrane preparations were done as described above.

**Functional Characterization of Chimeras and Site-Directed Mutants.** CHAPS-solubilized *E. coli* membrane preparations were used directly in steroid hydroxylase assays as described previously (Born et al., 1996; Fraser et al., 1997). Ten picomoles of P-450 were reconstituted with 40 pmol of *E. coli*-expressed rat NADPH-P-450 reductase, 10 pmol of rat cytochrome $b_5$, and 0.1 mg/ml dioleoylphosphatidylcholine and 0.06% CHAPS in a minimal volume. Assays were performed for 10 min at 37°C in 15 mM $MgCl_2$, 50 mM HEPES buffer (pH 7.6), 0.06% CHAPS, and 1 mM NADPH. Reactions were stopped with the addition of 50 $\mu$l tetrahydrofuran to each reaction tube. $^{14}$C-Steroid stock solutions were made in 100% methanol. Care was taken so that methanol concentrations in the reaction mixture were equivalent and did not exceed 1% of the total reaction volume. Individual assays were performed using concentrations of testosterone, progesterone, and androstenedione ranging from 25 to 250 $\mu$M. Hydroxysteroid metabolites were identified by relative mobility on thin-layer chromatography and by comparison with authentic standards.

## Results

**Generation of Chimeras of 3A12 and 3A26.** *Dra*III, *Pst*I, and *PpuMI* restriction endonuclease sites used in the generation of hybrid 3A12/3A26 enzymes are outlined in Fig. 2. These sites were instrumental in separating small groups of amino acid residue differences between 3A12 and 3A26 and characterizing the contributions to alterations in steroid hydroxylase activity. Progesterone 6$\beta$-hydroxylation was chosen as the marker activity for these studies, because it discriminates best between 3A12 and 3A26. Specifically, restriction endonuclease fragments from 3A26 were inserted into 3A12, and the resulting hybrid proteins were examined for loss of progesterone hydroxylase activity. Any major reductions in activity would indicate amino acid residue differences within the exchanged fragments that contribute to differential activity displayed by 3A12 and 3A26. Site-directed mutagenesis was then used to identify which of the incorporated residue differences might be responsible for alterations in catalytic activity.

| Mutant | Orientation | Primer Sequence |
|---|---|---|
| S368P | fwd. | 5'-ACTCTCCGATTATACCCAGTCGCTGGTAGACTTGAG-3' |
| S368P | rev. | 5'-CTCAAGTCTACCAGCGACTGGGTATAATCGGAGAGT-3' |
| V369I | fwd. | 5'-ACTCTCCGATTATACTCAATCGCTGGTAGACTTGAG-3' |
| V369I | rev. | 5'-CTCAAGTCTACCAGCGATTGAGTATAATCGGAGAGT-3' |
| S368P V369I | fwd. | 5'-ACTCTCCGATTATACCCAATCGCTGGTAGACTTGAG-3' |
| S368P V369I | rev. | 5'-CTCAAGTCTACCAGCGATTGGGTATAATCGGAGAGT-3' |
| I129M | fwd. | 5'-AAGAGAATGCGAACTTTGCTGTCT-3' |
| I129M | rev. | 5'-CAAAGTTCGCATTCTCTTCCACTC-3' |
| I187T | fwd. | 5'-ACCAGCACATCGTTTGGAGTGAACATTG-3' |
| I187T | rev. | 5'-AAACGATGTGCTGGTAATCACATCCATGCTGTAGG-3' |
| N209K | fwd. | 5'-ACCAAGAAGCTCTTAAAATTTGATTTCCTTGAC-3' |
| N209K | rev. | 5'-TTTTAAGAGCTTCTTGGTATTTTCCACAAACGG-3' |
| L220F | fwd. | 5'-CCATTTTTCTTCTCCATATTACTG-3' |
| L220F | rev. | 5'-GAAGAAAAATGGGTCAAGGAAATC-3' |

**Fig. 1.** Primers used for site-directed mutagenesis. Mutation generated is listed in left column, and primer orientation is indicated in second column. Matching forward and reverse primer sequences are listed with base pair alterations underlined.

In Fig. 2, the 6$\beta$-hydroxylase activity of the hybrid enzymes is presented as a percentage of 3A12 wild-type activity. The chimeric enzymes A and B, generated with *Dra*III, separate the first 4 amino acid differences from the remaining 18 and exhibited decreased activity when compared with wild-type 3A12. As seen with hybrid B, a 66% decrease in activity resulted from the presence of only four 3A26 residues in the N-terminal region.

The second set of hybrids, C and D, was generated using a *PpuMI* site that separates the final 8 amino acid residue differences from the 14 upstream changes. Chimera C retained 75% of the 6$\beta$-hydroxylase activity of 3A12 despite the replacement of eight C-terminal residues with those of 3A26. However, construct C no longer exhibited any 16$\alpha$-hydroxylase activity (data not shown), indicating that some differences in stereo- and regioselectivity did result from these alterations. Overall, the data from construct C indicated that the final eight amino acid residue differences were not major contributors to the 6$\beta$-hydroxylase activity of 3A12. Additionally, chimera D exhibited only 2% of 3A12 activity, indicating that the C-terminal differences were not sufficient to confer progesterone hydroxylase activity on 3A26.

An internal 450-bp *Pst*I fragment containing six amino acid residue differences was then exchanged between the two wild-type constructs, generating E and F shown in Fig. 2. The

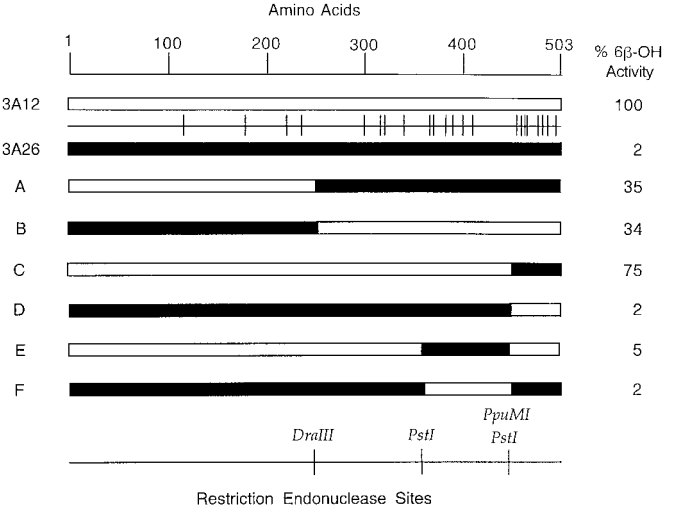


**Fig. 2.** Hybrid constructs of canine P-450 3A12 and 3A26. 3A12 is shown with white bars and 3A26 is shown in black bars. Hatched lines between wild-type 3A12 and 3A26 represent the relative positions of the 22 amino acid residue differences between the two enzymes. Left column identifies each hybrid according to the nomenclature used in text. Hybrids are represented by segments corresponding to each of the parent proteins. Restriction endonuclease sites employed in chimera generation are indicated at the bottom. Right column represents percent of 3A12 6$\beta$-hydroxyprogesterone formation.

activities of these chimeras were both extremely low, representing only 2% and 5% of 3A12 activity. These results indicated that residues both within and outside of the *Pst*I fragment contribute to the 6β-hydroxylation of progesterone. Significantly, the activity of the 3A12 hybrid E containing these six changes dropped by 95%, indicating that essential residue differences could be found in this region.

**Site-Directed Mutagenesis of Hybrid Constructs.** The results obtained with hybrid 3A12/3A26 proteins primarily implicated two regions of 3A12, the N-terminal and internal *Pst*I regions, as being important for 6β-hydroxylation of progesterone. The next set of experiments employed site-directed mutagenesis of individual and multiple codons encoding residue differences within the regions that were predicted to play roles in the observed catalytic variability of 3A12 and 3A26. Based on a number of previous reports, substrate recognition sites (SRSs) for the 3A enzymes are similar to those reported for the family 2 enzymes (Gotoh, 1992; Szklarz and Halpert, 1997; Harlow and Halpert, 1997; Domanski et al., 1998). As such, alterations were made with special consideration of differences that fall within the putative family 3 SRS regions.

The six differences in amino acid sequence in the *Pst*I fragment are found at residues 344, 348, 368, 369, 400, and 406. Of these, residues 368 and 369 fall within the proposed SRS5 of the 3A enzymes (Szklarz and Halpert, 1997). Construct E was used as template for the conversion of Ser-368 and Val-369 of 3A26 to Pro and Ile of 3A12, respectively. These changes were made individually and in combination. The results presented in Fig. 3A indicated that the substitutions regenerated 25 to 40% of the 6β-hydroxylase activity independently and acted together in the regeneration of 98% of the activity of wild-type 3A12. The restoration of activity by the back-mutation of residues 368 and 369 to those found in 3A12 showed that both of these residues are required for high progesterone hydroxylase activity and may play significant roles in conferring activity upon 3A26.

Construct B was used in similar studies presented in Fig. 3B. Single mutations were generated at residues 129, 187, 209, and 220 in chimera B. The only residue difference of the four targeted amino acids found to be within an SRS (SRS2) was 209. Interestingly, the Ile-187 → Thr change restored the activity of the chimera back to that of 3A12, whereas the changes at residues 129, 209, and 220 had no effect on the ability of the chimeric mutants to hydroxylate progesterone.

**Site-Directed Mutagenesis of 3A26.** The findings from hybrid and hybrid/mutant studies indicated that residues 187, 368, and 369 contribute significantly to the differences in 6β-hydroxyprogesterone production by 3A12 and 3A26. It was therefore of interest to determine whether the conversion of these three residues in 3A26 to the corresponding 3A12 residues would confer 3A12-like activities. Using the same *Dra*III and *Pst*I restriction endonuclease sites, fragments containing the mutant regions were back-cloned into the wild-type 3A26 construct. The single I187T mutant construct did not express well and was not employed in steroid hydroxylase assays. The data from steroid hydroxylase assays of the S368P/V369I double mutant and I187T/S368P/V369I triple mutant are presented in Table 1. The double mutant was shown to recover 20% of the 3A12 6β-hydroxylase activity and the triple mutant 36%. In comparison, less of the 16α-hydroxylase activity (16% and 18%, respectively) was regenerated by these substitutions. These data demonstrated that residues 187, 368, and 369 are major contributors to the differences in 6β-hydroxylation of progesterone observed between 3A12 and 3A26.

**Hydroxylation of Testosterone and Androstenedione.** Based on the findings with progesterone, it was of interest to examine the catalytic profiles of the 368/369 double and 187/368/369 3A26 triple mutants with testosterone and androstenedione as substrates. These findings (Table 1) showed that the triple mutant catalyzed the formation of 6β-hydroxytestosterone and 6β-hydroxyandrostenedione at the same rate as 3A12, whereas the double mutant displayed lower activity. Interestingly, the rates of 2β- and 15β-hydroxytestosterone and of 16β-hydroxyandrostenedione formation by the triple mutant were higher than those of wild-type 3A12. The results indicated that residues 187, 368, and

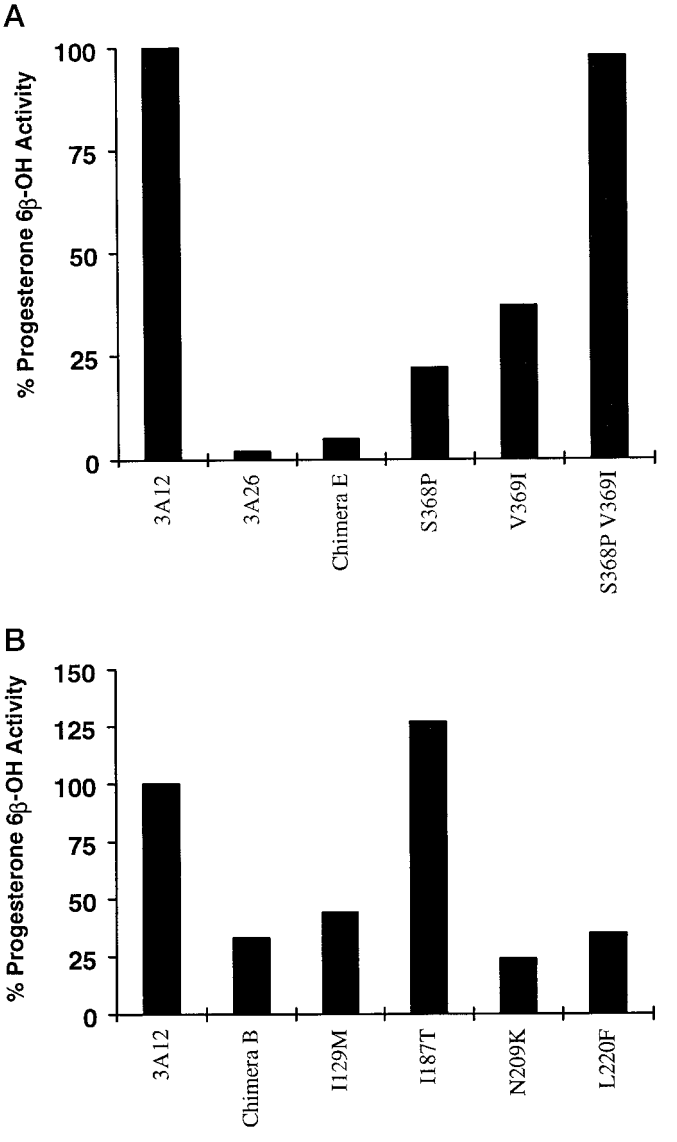


**Fig. 3.** Site-directed mutagenesis of chimeras. Mutagenesis of 3A12/3A26 chimeras was used to restore rates of 6β-hydroxylation of progesterone to those of wild-type 3A12. Progesterone hydroxylase rates are shown as a percentage of wild-type 3A12 activity. A, mutants were generated from chimera E by mutating residues 368 and 369 together as well as individually. B, alteration of chimera A by converting residues 129, 187, 209, or 220 from 3A26 to 3A12 individually.

369 are the major contributors to the differences in 6β-hydroxysteroid production by canine P-450 3A12 and 3A26.

**Multiple Hybrid Constructs.** Because the 3A26 triple mutant exhibited only one-third of the progesterone 6β-hydroxylase activity of 3A12 and gave different metabolite profiles with all three steroids tested, a set of multiple hybrid and hybrid/mutant enzymes (G, H, J, K, and L) was generated using a combination of *PpuMI* and *Pst*I restriction fragments of wild-type and mutagenized constructs (Fig. 4). Construct G combined the four N-terminal residue differences from 3A12 with the SRS5 Pro-368 and Ile-369 residues of 3A12, forming a hybrid/mutant that exhibited 70% of the progesterone 6β-hydroxylase activity of wild-type 3A12. Chimera H combined the same N-terminal four residue differences from 3A12 with the internal *Pst*I fragment containing six residue differences from 3A12, including residues 368 and 369. Interestingly, the additional four 3A12 residues in the *Pst*I region compared with construct G reduced the rate of 6β-hydroxyprogesterone formation to 38% of 3A12. This unexpected result suggested that residue-residue interactions involving the *Pst*I region might play a role in the steroid hydroxylase rate differences between 3A12 and 3A26.

Further evidence for such interactions was found in the examination of hybrids J and K. Hybrid J is a combination of the N-terminus of 3A26 (containing 8 residue differences) and the C-terminus of 3A12, containing 14 differences. In comparison, chimera K consists of the N-terminal portions of 3A12 and the C-terminal regions of 3A26. Hybrid J displayed only 19% of 3A12 progesterone 6β-hydroxylase activity. In addition, the ability of chimera K to 6β-hydroxylate progesterone was lower than that of the previously described chimera A, in which four fewer N-terminal 3A12 residues were observed to generate more than a third of 3A12 activity. Overall, the data in Figs. 2 and 4 indicated that the region from the *Dra*III site at codon 268 to the *Pst*I site at codon 331 and from the *Pst*I site at codon 331 to the *PpuMI* site at codon 459 must originate from the same enzyme for optimal activity. The possibility of residue-residue interactions between these two regions is supported by our molecular modeling of human 3A4, which indicates that residues 312, 313, 368, and 369 are within 4 Å of one another.

The possibility of residue-residue interactions suggested that other regions of 3A12 and 3A26 might play roles in the differences observed in their catalytic profiles. Previous studies of rabbit 2C2 and 2C1 indicated that the C-terminal 28 amino acid residues played important roles in the generation of progesterone hydroxylase activity (Ramarao et al., 1995; Ramarao and Kemper, 1995). Therefore, the C-terminal *PpuMI* fragment of 3A12 containing eight residue differences was cloned into the 3A26 I187T/S368P/V369I triple mutant to yield construct L (Fig. 4). This construct exhibited 77% of the progesterone 6β-hydroxylase activity of 3A12, which is twice as high as the triple mutant alone (Table 1). The results indicate that residues in the C-terminus act in concert with residues 187, 368, and 369 to convert the low progesterone 6β-hydroxylase activity of 3A26 to the high activity of 3A12. Construct L retained high activity for testosterone and androstenedione 6β-hydroxylation and showed product ratios with all three steroids intermediate between 3A12 and the 3A26 triple mutant (Table 2).

**Human 3A4 Mutagenesis and Catalytic Profiles.** The finding that residue 187 contributes significantly to the ability of canine cytochromes 3A to catalyze steroid hydroxylations has some bearing on human P-450 3A4 activity. Except for 3A26, a Thr residue is found at this position in all mammalian 3A enzymes including 3A4. Site-directed mutagenesis was used to convert Thr-187 in 3A4 to Ile to examine the role of this residue in steroid hydroxylations. The data from these studies are presented in Table 3 and show a reduction in activity similar to that observed for the canine enzymes. The alteration, which occurs outside of any of the proposed SRSs, is intriguing and may be of interest in future investigations of human 3A enzymes.

## Discussion

The results presented here describe for the first time the use of highly related but functionally distinct P-450 3A in structure-function analyses of this important class of enzymes. The design of these experiments involved the concerted use of chimeras and site-directed mutants to identify specific residues that contribute to the observed catalytic differences between 3A12 and 3A26. The basic approach of introducing a loss of 3A12 function through the insertion of 3A26 restriction fragments into 3A12 coupled with back mutations to restore 3A12-like activity was a useful strategy in these studies of two highly related enzymes.

The identification of residues 187, 368, and 369 as major contributors to the steroid hydroxylase activity exhibited by 3A12 and 3A26 resulted in the generation of a 3A26 triple mutant that displayed certain catalytic activities similar to those of 3A12. A 20-fold increase in progesterone 6β-hydroxylase activity was observed for the triple mutant when compared with the 3A26 wild-type, along with 10-fold increases in rates of 6β-hydroxytestosterone and 6β-hydroxyandrostenedione production. It is interesting to note that the 3A26 triple mutant regained more progesterone 6β-hydroxylase than 16α-hydroxylase activity. Differences in the testoster-





TABLE 1

Steroid hydroxylase activities of P-450 3A12 and 3A26 double and triple mutants

Solubilized *E. coli* membrane preparations containing 10 pmol of 3A12, 3A26 S368P/V369I, or 3A26 I187T/S368P/V369I mutants were reconstituted with 40 pmol P-450 reductase and 10 pmol cytochrome $b_5$ and analyzed for progesterone, testosterone, and androstenedione hydroxylase activity. Substrate concentrations of 250 μM were used throughout. Rates are given in nanomoles product per minute per nanomole P-450 and represent mean of 2 to 4 incubations with two separate preparations of each enzyme. Numbers in parentheses represent 3A26 mutant activity as a percentage of 3A12 activity.

| P-450 | Progesterone | | Testosterone | | | Androstenedione | |
|---|---|---|---|---|---|---|---|
| | 6β | 16α | 15β | 6β | 2β | 6β | 16β |
| 3A12 | 5.8, 6.6 | 0.84, 0.91 | 0.21, 0.30 | 2.6, 3.5 | 0.63, 0.91 | 4.4, 4.5 | 0.53, 0.60 |
| 3A26 S368P/V369I | 0.70, 0.98 (14) | 0.14, 0.14 (16) | 0.22, 0.26 (94) | 1.4, 1.6 (49) | 0.53, 0.63 (75) | 1.6, 2.7 (48) | 0.30, 0.47 (73) |
| 3A26 I187T S368P/V369I | 2.1, 2.4 (36) | 0.16, 0.15 (18) | 0.69, 0.62 (257) | 3.4, 2.7 (100) | 1.7, 1.1 (182) | 3.7, 5.8 (107) | 1.1, 1.6 (239) |

one hydroxylase profiles were also identified between 3A12 and the 3A26 triple mutant. Thus, the mutant exhibited 2- to 3-fold higher 2$\beta$- and 15$\beta$-hydroxylase activities, but similar 6$\beta$-hydroxylase activity when compared with 3A12. The data are reminiscent of the effects of $\alpha$-napthoflavone on 3A4, where preferential stimulation of the 2$\beta$- and 15$\beta$-hydroxytestosterone products was observed compared with the 6$\beta$-hydroxy product (Harlow and Halpert, 1997). 3A12 and the 3A26 triple mutant, however, did not display any marked differences in relative stimulation of the various hydroxytestosterone products by $\alpha$-napthoflavone (data not shown). These findings indicate that the different product profiles of 3A12 and the 3A26 triple mutant reflect the orientation of the substrate in the active site rather than constitutive activation of the triple mutant.

A role for the 44 C-terminal residues of 3A12 in modulating the steroid hydroxylase activities and profiles of the 3A26 triple mutant was also demonstrated. Previous studies with rabbit 2C2 and 2C14 indicated the importance of the 28 C-terminal residues of 2C14 in increasing laurate hydroxylase activity and conferring testosterone 16$\beta$-hydroxylase activity (Uno and Imai, 1992). These authors suggested that a conformational change had been induced in the C-terminal hybrid, enhancing coupling efficiency between NADPH utilization and laurate hydroxylation. In a similar fashion, Ramarao et al. (1995) found that the 28 C-terminal residues of 2C1 can confer progesterone 21-hydroxylase activity on 2C2. The key substitution was subsequently shown to be replacement of Ser-473 of 2C2 with Val from 2C1 (Ramarao and Kemper, 1995). The last 44 residues of 3A12 and 3A26 exhibit 8 amino acid substitutions, 5 of which (positions 474, 476, 477, 479, and 480) are within SRS6. In particular, the Ser residue at position 474 is unique to 3A26, whereas other 3A enzymes, including 3A4 and 3A12 have a Pro at this position. The difference in local conformation caused by a Pro/Ser substitution could well alter the shape of the active site and affect substrate binding or coupling efficiency. The residue differences at the C-terminus of the canine P-450 3A are an excellent target for identifying additional determinants of the differences in catalytic activity observed between these enzymes.

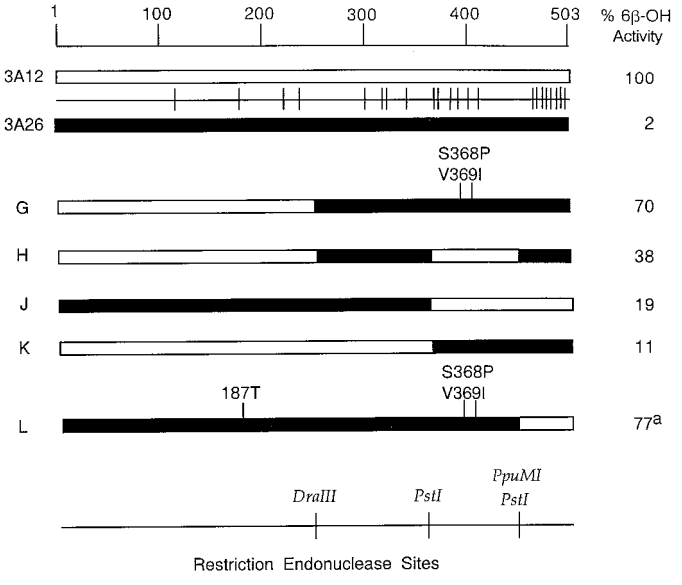


**Fig. 4.** Additional constructs used in identification of regions and residues responsible for differences in the 6$\beta$-hydroxylation of progesterone. 3A12 regions are noted with white bars and 3A26 regions are indicated by black bars. Changes generated by site-directed mutagenesis are listed above the construct modified. Progesterone 6$\beta$-hydroxylase rates are indicated in the column on the right and shown as a percentage of 3A12. [a]6$\beta$-hydroxylase activities of two separate preparations of chimera L were 5.7 and 4.0.

TABLE 2

Steroid hydroxylase metabolite ratios of P-450s 3A12, the 3A26 I187I/S368P/V369I triple mutant, and the 3A26 triple mutant with 44 carboxy-terminal residues from 3A12 (construct L).

Solubilized *E. coli* membrane preparations containing 10 pmol of wild-type 3A12, 3A26 I187T/S368P/V369I, or 3A26*PpuMI* mutant were reconstituted with 40 pmol P-450 reductase and 10 pmol cytochrome $b_5$ and analyzed for progesterone, testosterone, and androstenedione hydroxylase activity. Substrate concentrations of 250 $\mu$M were used throughout. These ratios were derived from 2 to 4 incubations on two independent enzyme preparations.

| P-450 | Testosterone | | Progesterone | Androstenedione |
|---|---|---|---|---|
| | 6$\beta$:2$\beta$ | 6$\beta$:15$\beta$ | 6$\beta$:16$\alpha$ | 6$\beta$:16$\beta$ |
| 3A12 | 4.0 | 12.0 | 7.0 | 7.3 |
| 3A26 I187T S368P/V369I | 2.2 | 4.6 | 14.4 | 3.4 |
| 3A26*PpuMI* I187T/S368P/V369I | 2.6 | 13.5 | 11.1 | 4.3 |

The importance of residue 187 for steroid hydroxylation by canine P-450 3A also has some precedent in a prior study of rabbit P-450 2C3. An Ile/Met difference at residue 178 in 2C3 alters the $K_m$ for progesterone and was proposed to influence the I-helix (Hsu et al., 1993). Ile-178 in 2C3 aligns with residue 184 in the E-helix in human and canine 3A enzymes. Our experiments did not indicate an alteration in $K_m$ of 3A4 T187I (data not shown); however, reductions in activity similar to those found in the canine 3A12/3A26 system were observed. Our molecular model of P-450 3A4 is consistent with an influence of residue 187 on the position of the I-helix situated just above it (Szklarz and Halpert, 1997). The inser-

TABLE 3

Steroid hydroxylase activities of human P-450s 3A4 and 3A4 T187I single mutant

Solubilized *E. coli* membrane preparations containing 10 pmol of either wild-type 3A4 or 3A4 T187I mutant were reconstituted with 40 pmol P-450 reductase and 10 pmol cytochrome $b_5$ and analyzed for progesterone, testosterone, and androstenedione hydroxylase activity. Substrate concentrations of 50 $\mu$M were used throughout. Rates are given in nanomoles product per minute per nanomole P-450 and represent mean of 2 to 4 incubations with two separate preparations of each enzyme. Numbers in parentheses represent mutant activity as a percentage of wild-type activity.

| P-450 | Progesterone | Testosterone | Androstenedione |
|---|---|---|---|
| | 50 $\mu$M | 50 $\mu$M | 50 $\mu$M |
| 3A4 | 8.6, 5.5 | 7.4, 5.7 | 2.7, 2.7 |
| 3A4 T187I | 3.7, 2.8 (46) | 2.7, 3.1 (44) | 1.3, 1.2 (46) |

tion of a much larger Ile in place of the native Thr may alter the positions of portions of the E- and I-helices, thus potentially affecting the active site of 3A4. These changes may contribute to a difference in substrate binding, access to the heme group, or access to the binding pocket.

A recent study of human 3A4 structure-function relationships involved two of the same amino acid substitutions identified as crucial for the differences in steroid hydroxylation between canine 3A12 and 3A26 (He et al., 1997). A Pro-369 to Ser substitution greatly decreased 3A4 expression in *E. coli*, whereas conversion of Ile-369 to Val in 3A4 decreased progesterone 16$\alpha$-hydroxylase activity by 4-fold and 6$\beta$-hydroxylase activity by 2-fold. Similarly, Ser-369 contributes to low activity and heterologous expression of 3A26, and conversion of Val-369 in 3A26 to Ile enhances progesterone hydroxylation. Based on three-dimensional modeling of 3A4 and analogy with family 2 enzymes, the residue differences between 3A12 and 3A26 in this region are likely to have a direct effect on the access of the substrate to the active oxygen or to the binding pocket. In general, the results of all site-directed mutagenesis studies to date indicate agreement among the studies with P-450 family 2 and family 3 enzymes in the identification of regions and specific residues important for catalysis.

In conclusion, this study has resulted in the identification of residues that play a role in differences in steroid hydroxylase activities of canine 3A12 and 3A26. Through the judicious use of chimeric constructs and site-directed mutants, the structure-function determinants of these two highly related but catalytically distinct enzymes were examined. Of the 22 amino acid differences between 3A12 and 3A26, three were found to be major contributors to the modulation of their catalytic activity. The insertion of 3A12 residues 187, 368, and 369 into 3A26 resulted in a 10- to 20-fold increase in the ability of 3A26 to hydroxylate steroids. All three positions are important for the function of human P-450 3A4 as well, which underscores the importance of canine studies as a model for human drug metabolism. These studies of highly related 3A enzymes from the same species exhibiting widely varying substrate specificities should be invaluable in the design of future studies involving this important class of enzymes.

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