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The Rat 17α-Hydroxylase-17,20-desmolase (CYP17) Active Site: Computerized Homology Modeling and Site Directed Mutagenesis

Ellen Buczko, YoungChul Koh, Yasushi Miyagawa and Maria L. Dufau*

Section on Molecular Endocrinology, Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, U.S.A.

A homology model of the rat 17α-hydroxylase-17,20 desmolase (CYP17)† steroid binding domain was derived from the α/βF supersecondary structural element of the 3α/20β hydroxysteroid dehydrogenase (HSD) of *Streptomyces hydrogenans* that constitutes a major segment of the C19 steroid binding cavity. A CYP17 arginine-rich domain, including Arg346, Arg361 and Arg363, that has previously been shown to be important to CYP17 catalytic activity, is conserved in this HSD structural element between two HSD domains known to be important to C19 steroid binding. These two HSD motifs, in addition to a C-terminal domain at the apex of the steroid binding cavity, are also present in similar though not identical forms in the rat CYP17 sequence. The model was evaluated in terms of both hydroxylase/lyase activity and stability of CYP17 mutant proteins (Tyr334Phe, Phe343Ile, Arg357Ala, Arg361Ala, Asp345Ala), and further tested with mutagenesis of Glu353, Glu358, and Tyr431. Those amino acids located at folding junctions in the model steroid binding domain (Glu358, Arg361, and Tyr431) are each individually required to prevent degradation of the nascent protein, as well as for basic hydroxylase/lyase activity. Genomic analysis of the rat CYP17 gene reveals that this domain is contained in exon 6, and a correlation exists between the length of exon 6 and the boundaries of the HSD supersecondary element. These studies demonstrate that exon 6 of the rat CYP17 is essential for CYP17 activity, and may be structurally related to the NAD-linked prokaryote α/βF supersecondary element.

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INTRODUCTION

The rat *P*450 cytochrome CYP17 [1] catalyzes two consecutive oxidation reactions on either the Δ4 or Δ5 steroid resulting in hydroxylation at the C17 carbon to produce the 17α-hydroxylated intermediate and cleavage of the C20,21 carbons to the androgen products, dehydroepiandrosterone or androstenedione. Characterization of the putative active site of the rat CYP17 by site directed mutagenesis has revealed that the "conserved domain" [2] between amino acid positions 343 and 363 carries a number of charged residues that contribute to hydroxylase and lyase activity [3, 4]. In particular, Arg346 has been shown to be essential for both Δ4 and Δ5 lyase, but not hydroxylase activity [3], Arg363 is of importance to the Δ4 hydroxylase reaction [3, 5] and Phe343 enhances Δ4 but not Δ5, lyase activity, and has no effect on hydroxylase activity [4]. These experiments suggested a separation of the hydroxylase and lyase activities, with either two active site domains or a single catalytic site with the substrate changing its position relative to the protein following the initial hydroxylase activity, perhaps anchored by the 17α-hydroxyl group of the steroid intermediate. Other investigations have shown a strict stereospecificity for 17α- rather than 17β-hydroxylation [6], and competitive inhibition between the substrate and its hydroxylated intermediate [7].

Our investigation of the steroid binding domain of the CYP17 began with defining a structural model for the region of the CYP17 that we found to be essential for catalytic activity through site directed mutagenesis.

*Correspondence to M. L. Dufau.

†CYP17 = *P*450c17; cytochrome $P450_{17\alpha}$; 17α-hydroxylase-17,20 desmolase (or lyase).

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Corresponding aligned regions of the Arg rich domain are not near the camphor binding site of the *P*450cam [8] in reported homology models [9, 10], or the fatty acid substrate binding cavity of the *P*450BM3 [11], and we searched for suitable alignments with C19 steroid binding domains in the Brookhaven Protein Databank. A significant sequence similarity of the CYP17 domain 334 to 368, (with identities at the essential amino acids Arg346, Arg361 and Arg363) exists to the α/βF structural domain of the streptomyces $3\alpha/20\beta$ dehydrogenase steroid binding cavity [12] resolved to 2.6 Å by X-ray crystallography [13]. The model was tested with additional site directed mutagenesis, and revealed a class of charged amino acids that were essential for protein stability, as well as hydroxylase/lyase activity.

MATERIALS AND METHODS

Homology model

X-ray coordinates of the α-carbon trace of the $3\alpha/20\beta$ dehydrogenase (1 hsd) from *Streptomyces hydrogenans* [13] was obtained from the Brookhaven Protein Databank online through the NIH network and was transferred into Quanta Version 3.3 (Molecular Simulations Inc, Waltham, MA). The 17α-hydroxylated intermediate was built from the crystal structure of progesterone (Cambridge Crystallographic Database) in ChemNote to the 17α- and β-hydroxylated forms. The homology model was based on regions of known importance to the active site in both the $3\alpha/20\beta$ HSD and the CYP17. A monomeric subunit from the HSD tetramer was used to build a homology model of the CYP17 steroid binding domain in the molecular modeling package Quanta. The dehydrogenase was edited to delete the amino end from amino acid 1 to 141 including the NAD binding domain, leaving a protein segment from amino acid 142 to the carboxyl end at 255 [12]. Other alignments were performed with the Fastscan subroutine in PCGene (Intelligenetics, Mountain View, CA), and GCG GAP subroutine (University of Wisconsin, Madison, WI). The homology model was created from the edited (see results) template crystal structures using the copy subroutine. Side chains were built and regularized using CHARMm RTF files, and optimized through energy minimizations to a local minima. Extensive changes in conformation were rejected in order to maintain the template geometry.

Site directed mutagenesis

The rat CYP17 cDNA was subcloned into a human cytomegalovirus promoter driven expression vector pCMV4 (gift from Dr D. Russell, University of Texas Southern Medical Center) as previously described [3]. Mutants were constructed using the recombinant circle polymerase chain reaction [14] with the normal(wild type) full-length CYP17 cDNA in pCMV4 as template. Synthetic oligonucleotides (Loftstrand Labs Limited) were designed to replace individual nucleotides within designated codons that result in the desired amino acid substitutions. The PCR reaction was carried out as previously described [3]. Each product was purified, and transfected into *E. coli* and the construction was verified by sequencing (U.S. Biochemicals, Sequenase Ver. 2.0) and reversal of mutant back to wild type

Expression of mutant and wild type CYP17 in COS1 cells

All wild-type and mutant DNAs in pCMV4 expression vector were transfected into COS1 cells by lipofectin method (Bethesda Research Laboratories, Life Technologies, Gaithersburg, MD) according to manufacturer's protocol. Briefly, after 72 h, 10^6 cells were incubated with the radiolabeled substrates (0.1 mCi; 0.2 mM) [^{14}C]pregnenolone, [^{14}C]progesterone, [^{14}C]17α-hydroxyprogesterone and [^{14}C]17α-hydroxypregnenolone in 2 ml of DMEM (Dulbecco's Modified Eagle's Medium) containing 10% fetal bovine serum at 37°C for 1 h. Steroids were extracted with ethyl acetate, concentrated by evaporation under N_2, and separated on thin layer chromatography (TLC) plates with chloroform/ethyl acetate (3:1). The radiolabeled substrates [^{14}C]pregnenolone, [^{14}C]progesterone, [^{14}C]17α-hydroxyprogesterone were purchased from Dupont-New England Nuclear. [^{14}C]17α-hydroxypregnenolone was obtained with organic extraction of the Arg346Ala CYP17 transfected COS1 cell, since an accumulation of the 17α-hydroxylated intermediate from an inhibition of the second lyase reaction was evident [3]. The [^{14}C]-steroids were separated on TLC and identified by autoradiography. The [^{14}C]17α-hydroxypregnenolone band on the TLC plate was scraped and extracted with ethanol. Authenticity of the labeled steroid was confirmed by crystallization of this product. Quantitation of each radioactive product was performed by the AMBIS Radioanalytic Imaging system (AMBIS System, Inc., San Diego, CA). Apparent k_{cat} and K_m for the hydroxylase and lyase activities was calculated with ENZFITTER (Elsevier BIOSOFT, Amsterdam, The Netherlands) as previously described [3]. All experiments were performed at least three times.

Western blot analyses of mutant and wild type CYP17

The microsomal fraction from approx. 5×10^7 transfected cells was isolated by centrifugation as previously described [15, 16], and applied to 10% SDS–acrylamide gel. Following transfer to nitrocellulose (BIO-RAD, Richmond, CA), the CYP17 was probed with a 1:1000 dilution of polyclonal rabbit antibody raised against an antigen consisting of the rat CYP17 amino acid sequence 187–201 peptide conjugated to thyroglobulin. IgGs from the antisera preparation were selected prior to use with the Absorbent^{IM}G affinity membrane disk (Genex Corp.,

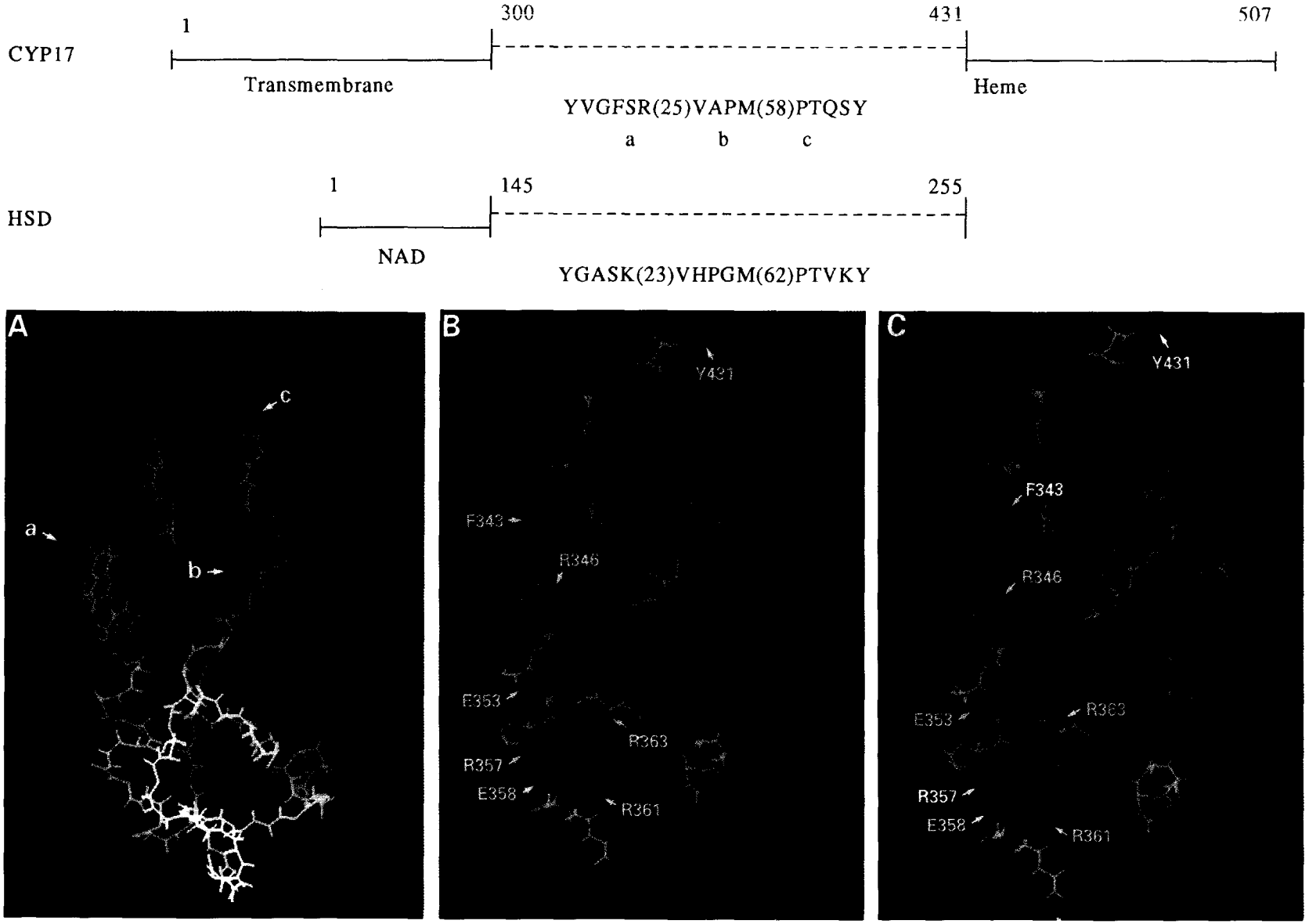

Fig. 1. (Top) Comparison of the linear sequence of CYP17 with three motifs from the active site of the HSD. Composite of functional domains of the rat CYP17, including the transmembrane (amino acid 1 to 21, and putative domain 169 to 186) and heme domains as defined in (1), and $3\alpha/20\beta$ HSD [12]. (bottom) Peptide backbone of the homology model of the CYP17 using the coordinates of the $3\alpha/20\beta$ HSD in the aligned region between CYP17 amino acid 300 and 431 (Fig. 2). (A) Domain a—YGFSR; b—VAPM; c—PTQSY. (B and C) Defined mutated amino acids—red, 17α-hydroxyprogesterone substrate—blue, and substrate oxygens—pink.

Gaithersburg, MD). Blots were incubated with CYP17 peptide antibody for 1 h at 22°C, and subjected to three 5 min and two 15 min washes with phosphate buffer saline containing 0.1% Tween 20. Subsequently, blots were incubated with goat antirabbit antiserum conjugated to horseradish peroxidase (1:1000 dilution) for 1 h. Blots were developed with the Enhanced Chemiluminescence, ECL Detection Kit (Amersham Arlington Heights, IL). CYP17 was detected from mutant, wild type and control Leydig cells. Negative controls are microsomal fractions from cells that were transfected with expression vectors that do not carry the CYP17 insert.

Radioimmunoassay

CYP17 from the microsomal fraction of cells transfected with wild type and mutants CYP17 and control Leydig cells were quantitated by radioimmunoassay using polyclonal rabbit antisera raised against the rat CYP17 peptide antigen 187–201 as previously described [4]. Briefly, microsomes (~30 μg protein in 100 μl RIA buffer) were incubated with 100 μl of CYP17 antibody (1:3000 dil.) and 200 μl RIA buffer (50 mM sodium phosphate pH 7.4 containing 0.1% NP40, 1% BSA, and 24TIU Trasylol% (Aprotinin, Sigma) for 24 h at 4°C. Subsequently, 20,000 dpm of ^{125}I-labeled peptide (187–201) (specific activity 500 Ci/mmol), was added with further incubation for 24 h. Separation of antibody bound from free protein/peptide was performed by PEG precipitation using 600 μl of 25% PEG (MW 6000) solution in RIA buffer, following addition of 150 μl 1% gamma globulin. Precipitate was counted and sample values were derived from reference standard curves (ID_{50} of displacement of 0.153 pmol per assay tube). All samples were assayed at 3.75–30 μg protein at four point levels. Samples from cells transfected with wild type, mutant mRNA and from control Leydig cells displayed parallelism to the standard curve while samples from cells transfected with expression vector only showed no displacement.

Isolation and sequence of exon 6 and flanking intron domains

A rat liver genomic lambda GEM-11 library (Promega, Madison, MI) was screened by plaque hybridization with a random labeled full length rat CYP17 cDNA probe [1]. DNA containing exon 6 was identified by Southern hybridization, and positive isolates were digested by restriction enzymes SacI and XbaI, subcloned into pGEM4z (Promega) cloning vector, and sequenced by the dideoxy chain termination protocol.

Pulse chase experiment

Transfected COS-1 cells (10^7 cells) were cultured for 12 h as described above, and washed 3X with methionine free Dulbecco's modified Eagle's medium (DMEM)/fetal bovine serum. Cells were preincubated at 37°C in Met free DMEM, and pulse labelled with 870 kBq [^{35}S]methionine [Expre^{35}S S, ^{35}S Protein Labelling Mix (Dupont, Wilmington, DE)] for 45 min. at 37°C. The cold chase was performed at 37°C for indicated time intervals. Cells were washed, sonicated, incubated on ice for 30 min, and centrifuged. Wild type and mutant CYP17 were extracted from lysates with purified 187–201 CYP17 antibody (see above) overnight at 4°C. The antigen/antibody complex was precipitated with protein A-agarose beads (Gibco, BRL, Bethesda, MD), centrifuged, washed and was resolved by 10% SDS–PAGE. [^{35}S]CYP17 was detected and quantitated by PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

Computer homology model

The CYP17 domain that is essential for catalytic activity (343–363) exhibits significant similarity to the HSD α/βF structural element between and including two HSD domains noted to be of importance to C19 steroid binding [Fig. 1(A, B)]. A GAP alignment

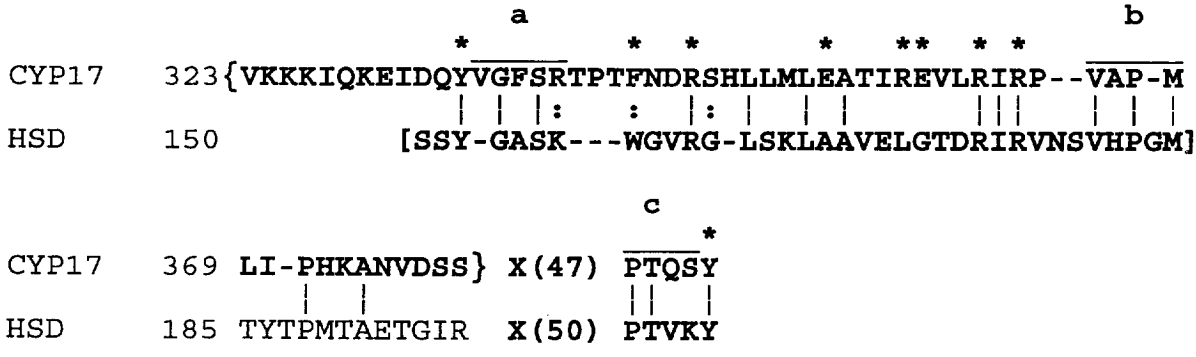

Fig. 2. Alignment of the rat CYP17 conserved domain (1) with regions listed as important for substrate binding [12] in the 3α/20 HSD, that was used to create the homology model of the CYP17 active site. { } CYP17 exon 6, * CYP17 substituted amino acids, [ ] HSD α/βF module, ¦ identity, : conserved substitution.

Table 1. CYP17 mutant activities (k_{cat}) relative to wild type

CYP17	Hydroxylase	Lyase
Wild	1.0	1.0
Y334F*	0.81 ± 0.01	1.08 ± 0.06
F343I**	1.02 ± 0.10	0.62 ± 0.09
R346A*	0.80 ± 0.20	0
E353A	0.97 ± 0.09	0.96 ± 0.08
R357A*	0.35 ± 0.02	0.14 ± 0.21
E358A†	0	0
E358Q	0	0
E358D	0.05 ± 0.002	0.05 ± 0.003
R361A†	0	0
R363A*	0.24 ± 0.10	0.69 ± 0.12
Y431A	0	0
Y431F	0.81 ± 0.06	0.88 ± 0.09

*Reported in [3].

**Reported in [4].

†Analyzed 6, 12, 24 and 48 h after transfection remaining mutants were analyzed 72 h after transfection.

between the rat CYP17 and HSD of this region displays a 70.4% similarity of identical and conservative replacements, and a 55.5% identity (Fig. 2). The aligned region constitutes approx. 94% of the α/βF supersecondary element (Fig. 2—in brackets), and is a structural domain of the HSD steroid binding region. The HSD distal domain c (PTVKY) is included in the functional HSD steroid binding cavity at its apex (Fig. 1), and a similar CYP17 motif (PTQSY) is located analogous to the HSD motif in the CYP17 linear sequence (Figs 1 and 2). The model is displayed as the substrate binding cavity [13], and represents the consecutive amino acids of α/βF supersecondary element as well as the PTXXY domain [Fig. 1(A)]. Only the amino acid domain from a to b, and at c exhibit significant similarity to the CYP17 (Fig. 2). The HSD and CYP17 sequence between domains b and c contain no obvious similarity, aside from the number of amino acids, and this domain is not part of the substrate binding cavity in the CYP17 model.

A major consideration for the model CYP17 steroid binding domain is the position of the steroid in the two consecutive reactions. Both imidazole inhibitor studies [17] and site directed mutagenesis [3, 4] indicate that protein contacts with the steroid change during the course of the two reactions. A competitive inhibition between the steroid and its hydroxylated intermediate has been shown and suggests a single enclosed binding cavity for the two reactions, with the C17/20 end proximal to the heme oxygen for the two oxidations. The Δ 4 and Δ5 steroid contains C3 keto/hydroxyl and C20 keto groups, and addition of the C17 hydroxyl group during the hydroxylase reaction gives a potential third bonding element on the steroid intermediate, that is not present on the primary steroid substrate. Amino acid contacts with the C17 hydroxyl group would be essential only for the second lyase activity that utilizes the 17α-hydroxyl steroid as substrate, and substitution of this amino acid would result in a differential loss of lyase, but not hydroxylase activity. Since, substitution of Arg346 for alanine resulted in this differential loss of lyase activity (Table 1), the C17 hydroxyl of the 17α-hydroxylated substrate in Fig. 1(B) was positioned adjacent to Arg346 in the model CYP17 binding cavity.

In positioning the 17α-hydroxyl group [pink—Fig. 1(B)] of the steroid intermediate at Arg346 in the model CYP17/HSD steroid binding cavity, the C3 βOH of the Δ 5 steroid would contact the opposing side of the cavity, and can be positioned proximal to Arg363. Substitution of Arg363 for Ala resulted in a substantial loss of 76% of the hydroxylase activity, with only a 30% loss in lyase activity (Table 1), and indicates that Arg363 may be important, though not essential for hydroxylase activity. Mutagenesis of none of the amino acids listed in Table 1 gave complete differential reductions in hydroxylase over lyase activity, similar to Arg346 for lyase over hydroxylase activity. Since the CYP17 catalyzes Δ4 and Δ5 hydroxylations with equal efficiency [4], the keto or hydroxyl group on the C3 carbon may not covalently interact with the protein. Recent studies [4] have identified Phe343 as a position that is of importance to lyase but not hydroxylase activity, and can discriminate between the Δ4 and Δ5 17-α hydroxy intermediates, indicating a change in C3 orientation between the two reactions [Fig. 1(C)]. However, movement of the steroid may rather reflect a change in the protein conformation during the heme oxidations. In that case, a single model may not be relevant for both reactions.

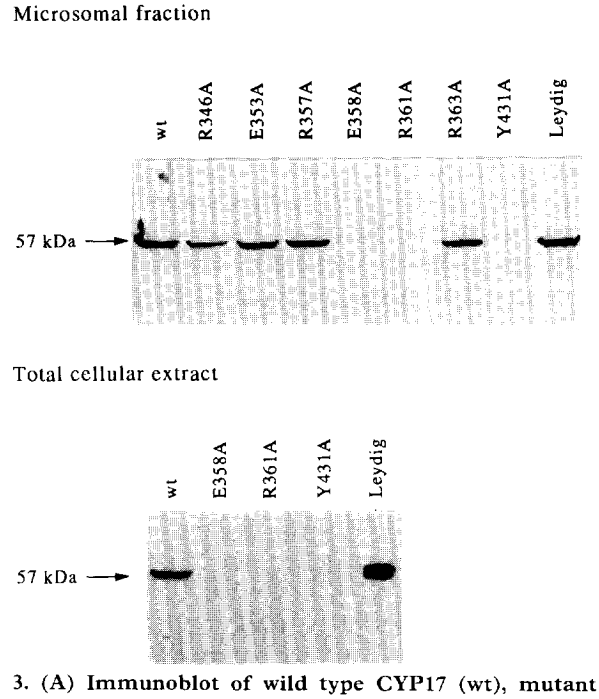

Fig. 3. (A) Immunoblot of wild type CYP17 (wt), mutant CYP17 and native CYP17 extracted from microsomal fractions of COS1 and Leydig cells after 72 h transfection. (B) Immunoblot of wild type, E358A, R361A and Y431A, and native CYP17 extracted from total cells; COS1 and Leydig cells after 72 h transfection.

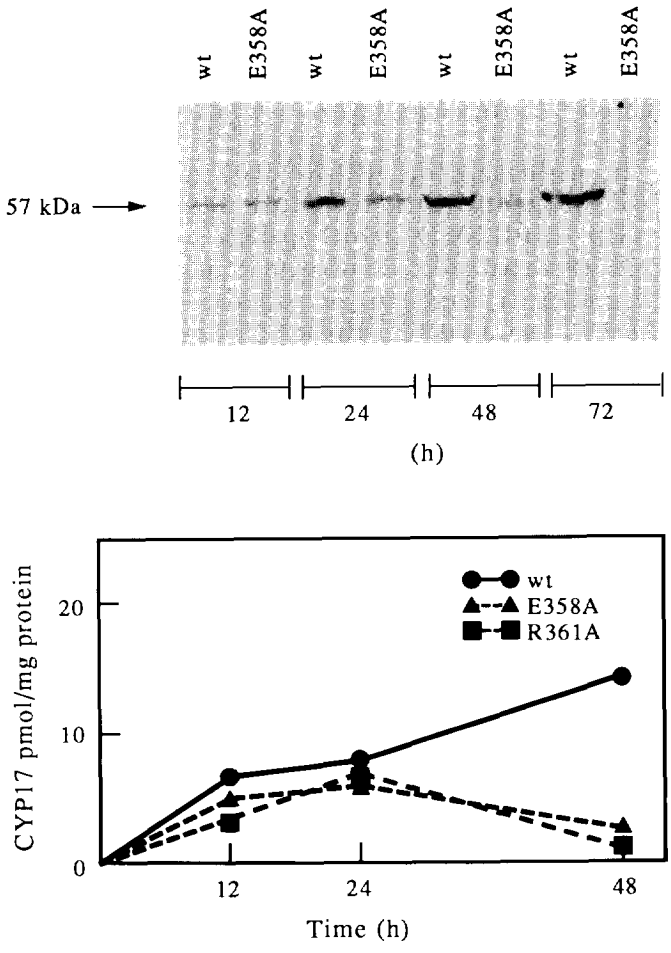

Fig. 4. (A) Immunoblots of wild type (wt) and E358A CYP17 extracted from whole cell homogenates of COS1 cells following 12, 24, 48 and 72 h transfections. (B) Microsomal levels of wild type (●), E358A (▲) and R361A (■) measured at 12, 24 and 48 h after transfection by RIA.

Based on this C19 steroid binding cavity that displays significant amino acid similarity to the essential Arg346 to Arg363 CYP17 domain, we identified several amino acids that may be of functional importance, either through steroid interaction or stabilization of the structural fold of the proposed binding cavity. These were tested with site directed mutagenesis.

Site directed mutagenesis

The hydrophilic amino acids Arg357, Glu358, Arg361, Glu353 and Tyr431 that line the substrate binding cavity of the model CYP17 were individually mutated to evaluate their functional roles. Substitutions of Glu358, Arg361 and Tyr431 with alanine totally abolished all hydroxylase and lyase activities (Table 1). Immunoblots using a polyclonal antibody raised against the sequence amino acid #187–201 [4] revealed that substitutions at these positions resulted in deviations in posttranslational processing. The polypeptide backbones of these mutant CYP17 were not present in microsomal [Fig. 3(A)] or total cellular [Fig. 3(B)] fractions after a 72 h transfection period. Thus, the absence of these mutants from microsomal fractions cannot be attributed to deviations in intracellular transport and localization. In addition, mRNA levels were equivalent to wild type, and mutant back protocols regenerated the active form of the CYP17 (see Experimental procedures). The CYP17 mutants, E358A, R361A, and Y431A, showed a pattern of premature degradation in transfection time studies (Fig. 4). These mutant CYP17 protein levels were close to wild type levels at 12 and 24 h incubations, although the mutant protein was inactive at these time periods (Table 1). However, a marked decrease at 48 h and total absence at 72 h relative to wild type levels was evident in immunoblots, and this was verified with RIA quantitation (Fig. 4). Pulse chase experiments reveal that substitution of either E358 or R361 or Y431 with alanine decreases the half life from greater than

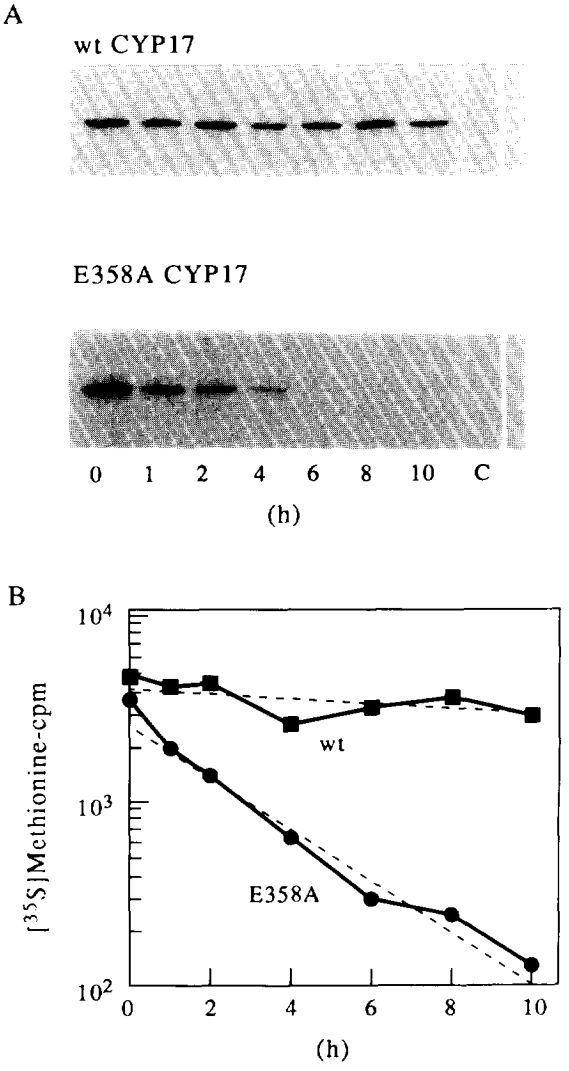

Fig. 5. (A) Pulse chase of wild type and E358A CYP17. 12 h transfection, 1 h pulse with [^{35}S]Met, followed by 0 to 10 h cold chase. SDS–gel electrophoresis of immunoprecipitated [^{35}S]Met CYP17. Control lane (C) is transfected vector without insert. (B) Semilog plot of actual cpm (●, ■) or exponential curve fit (- - -) R (E358CYP17) = 0.98, R (WTCYP17) = 0.72.

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aatagccccatttgggggagggcaggggcaatgcctggattcttctgtacagagataaccagagccctct
ctagctctcacacaagcagtggctgactctggctgtgggtaaaagttgacctatcctctagaaacaaggc
ctaggttacagagccgtcactggatagaatcattccatggtgttctgggtatgaccactgggctctgcta
agcccatgtctctcttagacttgaacaacctggtcatgtgacctggttctggcccacag

 967  GTG AAG AAG AAG ATC CAA AAG GAG ATT GAC CAG TAC GTA GGC
 323  Val Lys Lys Lys Ile Gln Lys Glu Ile Asp Gln Tyr Val Gly

1008  TTC AGC CGA ACA CCA ACT TTC AAT GAC CGG TCT CAC CTC CTC
 337  Phe Ser Arg Thr Pro Thr Phe Asn Asp Arg Ser His Leu Leu

1057  ATG CTG GAG GCC ACT ATC CGA GAA GTG CTG CGT ATC AGG CCG
 357  MET Leu Glu Ala Thr Ile Arg Glu Val Leu Arg Ile Arg Pro

1093  GTG GCT CCC ATG CTC ATC CCC CAC AAG GCT AAC GTT GAC TCT AG
 365  Val Ala Pro Met Leu Ile Pro His Lys Ala Asn Val Asp Ser Ser

gtacgcctttcttcccgagatatcagcctaggatgtagccactggctagcccactttgctcctcagacat
gttccacctggccttagttgctggtccacctagagtcaaacagtgactaccactgtcaatcaccctgccc
tagctactctatctacctccactgatacccactgactaagttgaattggcatcagcatctgg
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Fig. 6. Exon 6 from rat CYP17. Rat genomic sequence of exon 6 (upper case) and adjacent partial intron domains (lower case), accession #: U 14953.

10 h to 2 h (Fig. 5 and data not shown). It is unknown whether this decrease represents ER or lysosomal [18] degradation, or whether it involves induction of chaperone proteins and/or proteases [19].

Substitution of E358 with Asp resulted in a significant decrease in both hydroxylase and lyase activities of 95% when compared with wild type at 6 h after transfection. This substitution, however, did not result in total loss of activity as did the alanine substitution at this position, indicating that the extended length of the side chain of Glu relative to Asp as well as the negative charge are both important for hydroxylase and lyase activities. Substitution of Glu358 for Gln resulted in a mutant CYP17 that exhibited no activity (Table 1), confirming that the negative charge must be retained in the active protein (Table 1). This observation is consistent with the relative position of E358 in the model CYP17 at the bottom of the F loop that immediately precedes a change in the polypeptide direction, perhaps forming an extended ion pair with R361 that stabilizes the two sides of the F loop at the bottom of the CYP17 model cavity [Fig. 1(B and C)]. E358 and R361 are conserved in their respective linear positions in all of the known *P*450s [20, 21], although the *P*450s show preferences for different substrates, and may be examples of "determinants" [22] that define a folding pattern for a family of proteins with their substitution resulting in misfolding and degradation.

Substitution of amino acids that are present along the sides of the cavity in the model CYP17, Phe343, Glu353, Arg363, Arg357 and Arg346 [Fig. 1(B, C)] resulted in no deviations in microsomal CYP17 levels (Fig. 6), although the mutant proteins Arg363A, Arg357A, Arg346A and Phe343I did exhibit changes in hydroxylase and/or lyase activity [3, 4] (Table 1). The mutant CYP17 Arg346A, Arg363A and Phe343I exhibited differential reductions in hydroxylase or lyase activity, and therefore, it can be argued that these amino acids do not alter the primary fold of the protein for at least one of the reactions. Substitution of Arg357 for alanine resulted in significant decreases in both hydroxylase and lyase activities (Table 1), although substitution with lysine resulted in no changes in levels of these activities [3]. Thus, a positive charge is of primary importance at position 357, and its function may be correlated to the essential negative charge at position 358 (Glu vs Gln—Table 1).

Substitution of Tyr431 for alanine resulted in total loss of protein following a 72 h transfection (Fig. 3), though substitution of Tyr431 for phenylalanine did not reduce wild type activity (Table 1). Thus, only the aromatic ring at position 431, located at the apex of the HSD substrate binding cavity and model CYP17 [Fig. 1(B, C)] is essential for protein stability. In the dehydrogenase, this tyrosine has been proposed to be involved in substrate interaction through its hydroxyl group [13]. However, clearly the hydroxyl group of tyrosine is not required for activity in the CYP17 (Table 1). This is also consistent with our model since the hydroxyl group of Tyr431 is too distant from the docked substrate at Arg346 [Fig. 1(B)] to play a role in steroid binding. Similarly, the observation that either substitution of Glu353 or Phe334 does not reduce CYP17 activity (Table 1) is also consistent with the model CYP17 since these amino acids are not in the region of the C3 or C20 keto groups of the 17α-hydroxylated steroid docked at Arg346 in the homology model [Fig. 1(B, C)].

Although definitive evidence will have to await X-ray analysis, the results of mutagenesis of the amino acids listed in Table 1 are consistent with the proposed model of the hydroxylated intermediate docked at Arg346 in the HSD/CYP17 steroid binding cavity. Those mutations that result in total loss of activity and increased rates of degradation (E358 and R361) are at positions that appear to be essential for protein folding in the CYP17, and the corresponding model of the substrate binding site. In contrast, those mutations that result in differential or partial loss of enzyme activity do not affect the active site for at least one of the reactions, and appear to be important only for substrate or substrate intermediate interaction (Arg346 and Arg363).

Evolutionary considerations and overall conclusions

The fact that a similarity exists between the rat CYP17 Arg rich domain (which is conserved in most of the *P*450s) and a single structural element of the prokaryote HSD, indicates that the CYP17 may be another example of a modular protein [23], extracting its substrate binding domain from the HSD precursor. Analysis of the genomic sequence of the rat CYP17 (Fig. 6) shows that the domain between amino acid 329 and 379 that contains the putative steroid binding "conserved" [2] region and closely aligns with the complete α/βF element (Fig. 1), is conserved as a single exon 6 (Fig. 2). Exons have been correlated with supersecondary structural elements that correspond to structural domains [24], and the site of intron insertion within eukaryotic genes frequently lies between these domains [25].

This homology model is a first approximation that has been attempted because of the visible linear alignment of the CYP17 arginine rich region between domains a and b to the prokaryote HSD steroid binding cavity (Figs 1 and 2). However, motifs a, b and c have not been shown to be required for CYP17 hydroxylase/lyase activity. In former studies [3], substitution of Tyr334 (from the YGASK domain) for phenylalanine in the rat CYP17 (Table 1) did not affect either hydroxylase or lyase activities. In addition, Tyr334 is not conserved in the CYP17 across species (Fig. 7), so that this amino acid is apparently not a functional component of the CYP17 Δ5 binding domain. Similarly, Tyr431 (from the PTXXY domain) can be substituted by phenylalanine without loss of activity

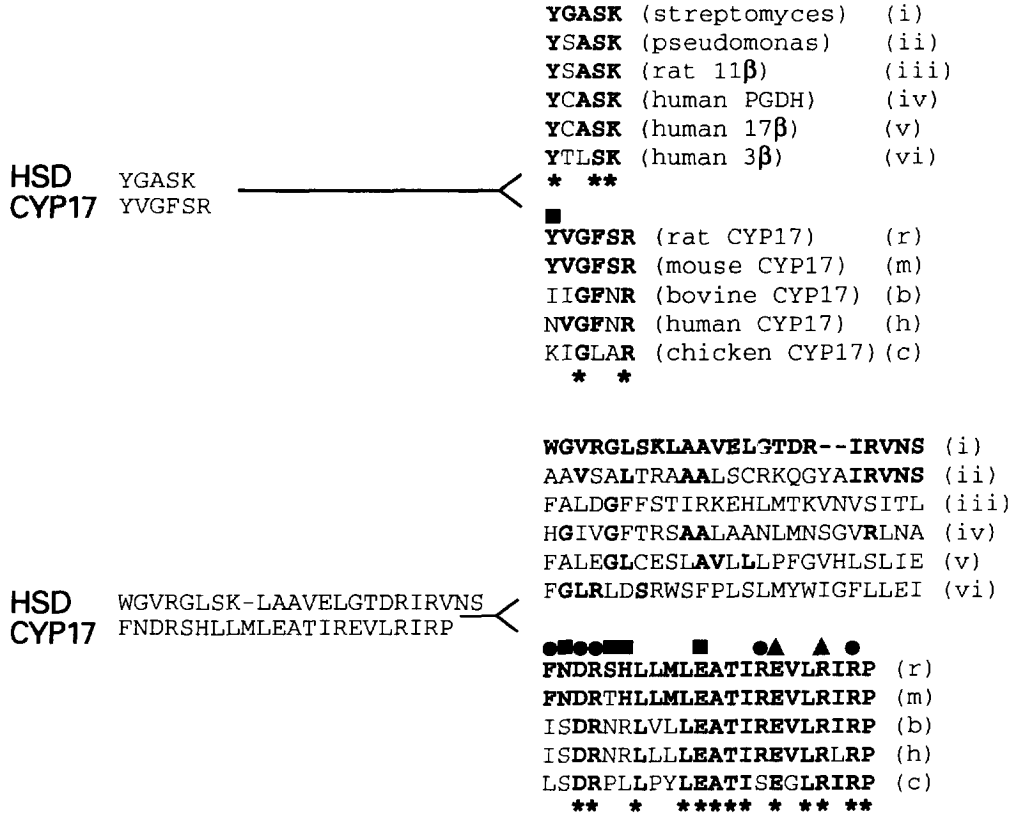

Fig. 7. Conservation of domains of proposed importance to substrate binding in the 3α/20β dehydrogenase α/βF element and the CYP17 exon 6. (i) 3α/20β HSD [12]; (ii) 3β HSD [29]; (iii) 11β dehydrogenase [30]; (iv) 15-hydroxyprostaglandin dehydrogenase [31]; (v) 17β HSD [32]; (vi) 3β HSD [33]; (r) rat CYP17 [1]; (m) mouse CYP17 [34]; (b) bovine CYP17 [35]; (h) human CYP17 [36]; (c) chicken CYP17 [37]. Mutations from refs. 3, 4 and this study. Residues indicated by symbols, are: ●, important for enzyme activity; ▲, important for maintaining protein levels; ■ do not contribute to enzyme activity. * indicate identity.

(Table 1). However, within the same α/β structural element, amino acids between Phe343 and Arg363, that are conserved only in the prokaryote HSD and the CYP17 (Fig. 7), have been identified by site directed mutagenesis as participating in the CYP17 oxidations. The apparent replacement of these amino acids later in evolution in the mammalian α and β dehydrogenases, suggests that this region does not participate in catalytic activity in the NAD linked dehydrogenases (Fig. 7). These observations indicate that a change in the binding position of the steroid within the prokaryote cavity appears to have taken place to accommodate the C17 oxidations, and movement of the steroid to the bottom of the α/βF structural element may have been necessary to precede the introduction of the heme in the CYP17. This hypothesis is consistent with the general observation that diverse proteins exhibit common protein folding patterns, and evolution involves replacement of the primary sequence within the conserved folds [22]. Our identification of a second class of mutations of highly conserved amino acids (Arg361, Glu358 and Tyr431) that result in the production of immunoreactive, inactive mutant CYP17 that is unstable and prematurely degraded (Figs 3, 4 and 5), suggests that clearance mechanisms have developed for mutations at important tertiary positions to insure that folding patterns are conserved, and misfolded proteins are not retained in the cell where they can acquire new functions.

In conclusion, the mosaic [26] design of the homology model, or the extraction of a supersecondary structural domain from the HSD appears to be a valid approach in the case of the CYP17, since amino acids from the conserved domain [2] that have been identified as important for catalytic activity or substrate binding [3, 4] are present at key docking positions in the model. The CYP17 functions as a protein complex, and overall reductions in both hydroxylase and lyase activities of mutant CYP17s that are not included in our alignment (Fig. 2) [27, 28], can be attributed to many factors from membrane insertion, to heme or flavoreductase binding, as well as the deviations in protein folding and stability that we have noted in this study. We have provided a working model of the putative steroid binding domain for determining mechanisms of substrate and inhibitor binding to the CYP17 during the hydroxylase and lyase reactions.

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