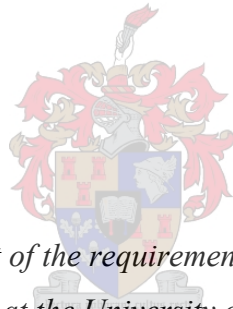


AN INVESTIGATION INTO THE CATALYTIC ACTIVITY OF PORCINE  
CYTOCHROME P450 17 $\alpha$ -HYDROXYLASE/17,20-LYASE

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*Thesis presented in partial fulfilment of the requirements for the degree Master of Science in  
Biochemistry at the University of Stellenbosch*

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Declaration:

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own original work and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

26 February 2014

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## ABSTRACT

In this study, the effect of the amino acid residues at positions 40 and 407 on the catalytic activity of porcine CYP17A1 was investigated. Porcine cofactor CYB5 was cloned from porcine liver tissue and its effect on the catalytic activity of porcine CYP17A1 was determined. The influence of rat, human and angora CYB5 on the lyase activity of porcine CYP17A1 was subsequently determined and compared to the influence of porcine CYB5. Wt porcine CYP17A1, which has residues Val40 and His407, catalysed the conversion of prog efficiently with ~50% prog converted to 17OHprog (~40%) and A4 (~10%) after 3 hr. After 24 hr, negligible levels prog remained with ~71% 17OHprog and ~25% A4 being produced. Low levels of 16OHprog were formed (~9%). The Leu105Ala mutation reduced wt 17 $\alpha$ -hydroxylase activity, with 70% prog remaining after 24 hr while 16OHprog (~10%) levels remained unchanged. Porcine CYP17A1 with residues Leu40 and His407, exhibited similar catalytic activity towards prog as did wt porcine CYP17A1 (Val40 and His407 residues), while porcine CYP17A1 with residues Leu40 and Leu407 increased the formation of A4 2-fold to 54% at 24 hr and porcine CYP17A1 with residues Val40 and Leu407 resulted in the highest formation of A4 (90%). Wt porcine CYP17A1, while having converted 95% of the prog substrate, produces only ~16% A4 after 24 hr. In the presence of porcine CYB5, however, the lyase activity was stimulated with 85% of prog being converted to A4 and only 13% 17OHprog remaining. The lyase activity was also stimulated by CYB5 from other species, resulting in an increase in A4 production of 60.6%, 24% and 11.6% by rat, angora and human CYB5, respectively. The degree of lyase stimulation correlated to the percentage identity of the CYB5 amino acid sequences to porcine CYB5. While the Val and Leu residues at position 40 do not appear to influence the lyase activity of porcine CYP17A1 as prominently as the residue at position 407, it is the charged residue at 407 that plays a significant role in the production of A4, decreasing A4 production irrespective of the Val and the Leu residues at position 40. It would, furthermore, appear that the stimulation of lyase activity of CYP17A1 is the greatest when assaying this activity in the presence of CYB5 of the same species as was detected when co-expressing porcine CYP17A1 and porcine CYB5.

## OPSOMMING

In hierdie studie is die invloed van die aminosuurresidue by posisies 40 en 407 op die katalitiese aktiwiteit van vark CYP17A1 ondersoek. Vark CYB5 is geklooneer vanuit vark lewer weefsel en die effek van hierdie kofaktor op die katalitiese aktiwiteit van vark CYP17A1 is bepaal. Die invloed van rot, mens en angora CYB5 op die liase aktiwiteit van vark CYP17A1 is daarna bepaal en vergelyk met die invloed van vark CYB5. Vark CYP17A1-VH, (kodeer Val40 en His407), kataliseer die omskakeling van prog doeltreffend met ~50 % prog wat omgeskakel word na 17OHprog (~40%) en A4 (~10%) na 3 uur. Na 24 uur, is feitlik alle prog omgeskakel, met ~71% 17OHprog en ~25% A4 geproduseer. Lae vlakke 16OHprog is ook gevorm (~9%). Die Leu105Ala mutasie verminder 17 $\alpha$ -hidroksilase aktiwiteit, met 70% prog wat na 24 uur nie omgesit is nie, terwyl 16OHprog (~10%) vlakke onveranderd gebly het. Vark CYP17A1-LH (kodeer Leu40 en His407), en CYP17A1-VH het dieselfde katalitiese aktiwiteit teenoor prog getoon, terwyl vark CYP17A1-LL (kodeer Leu40 en Leu407) die vorming van A4 2-voudig verhoog het tot 54% na 24 uur. Vark CYP17A1-VL (kodeer Val40 en Leu407) se katalitiese aktiwiteit het gelei tot die hoogste vorming van A4 (90%). Alhoewel CYP17A1-VH, 95% van die prog substraat omgeskakel het is slegs ~16% A4 geproduseer na 24 uur. In die teenwoordigheid van vark CYB5 is die liase aktiwiteit egter gestimuleer, en is 85% van die prog substraat omgeskakel na A4 met slegs 13% 17OHprog teenwoordig na 24 uur. Die liase aktiwiteit is ook gestimuleer deur CYB5 van ander spesies, wat lei tot 'n toename in A4 produksie van 60,6% , 24% en 11,6% deur rot, angora en menslike CYB5, onderskeidelik. Daar is gevind dat daar 'n sterk korrelasie is tussen die stimulering van die liase aktiwiteit en die persentasie aminosuur volgorde identiteit van CYB5 afkomstig vanaf die verskillende spesies. Terwyl die Val en die Leu aminosuurresidu op posisie 40 wel die liase aktiwiteit tot 'n mate beïnvloed, blyk dit uit die data dat die potensieel gelaai residue by 407 'n belangrike rol speel in die produksie van A4, en A4 produksie verlaag ongeag van die Val en die Leu residu by posisie 40. Dit wil ook verdermeer voorkom asof die stimulering van die liase aktiwiteit van CYP17A1 die hoogste is wanneer die ensiem gekataliseerde reaksie deurgevoer word in die teenwoordigheid van CYB5 en CYP17A1 afkomstig vanaf dieselfde spesies.



*Dedicated to my mother and father, I owe you everything but I will start with this.*

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Nothing is ever complete without the mention of my four-legged angels, my boys

*~Live, like someone left the gate open*

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## ABBREVIATIONS

16OHpreg	16 $\alpha$ -hydroxypregnenolone
16OHprog	16 $\alpha$ -hydroxyprogesterone
17OHpreg	17 $\alpha$ -hydroxypregnenolone
17OHprog	17 $\alpha$ -hydroxyprogesterone
3 $\beta$ HSD	3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta$ 5- $\Delta$ 4-isomerase
3D	3-dimensional
11 $\beta$ HSD	11 $\beta$ -hydroxysteroid dehydrogenase
17 $\beta$ HSD	17 $\beta$ -hydroxysteroid dehydrogenase
20 $\beta$ HSD	20 $\beta$ -hydroxysteroid dehydrogenase
A4	androstenedione, 4-androstene-3,17-dione
ACAT	acyl-coenzyme A: cholesterol acyltransferase
ACTH	adrenocorticotrophic hormone
adx/adr	adrenodoxin/adrenodoxin reductase
AKR1C3	aldo-keto reductase /17 $\beta$ HSD type5
An $\alpha$ / $\beta$	5 $\alpha$ -androst-16-en-3 $\alpha$ / $\beta$ -ol
andien $\beta$	androstadienol
androstanediol	5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol
androstanedione	5 $\alpha$ -androstane-3,20-dione
androstenediol	5-androstene-3 $\beta$ ,17 $\beta$ -diol
AR/ER	androgen/estrogen receptor
CAH	congenital adrenal hyperplasia
CBG	corticosteroid binding globulin
CPR	cytochrome P450 oxidoreductase
CYB5	cytochrome b5
CYP11A1	cytochrome P450 side-chain cleavage (P450scc)
CYP11B1	cytochrome P450 11 $\beta$ -hydroxylase
CYP11B2	cytochrome P450 aldosterone synthase
CYP17A1	cytochrome P450 17 $\alpha$ -hydroxylase/17,20-lyase
CYP19	cytochrome P450 aromatase
CYP21A2	cytochrome P450 21-hydroxylase
CYP2C5	cytochrome P450 21-hydroxylase
CYP46A1	cholesterol 24-hydroxylase
DHEA	dehydroepiandrosterone, 5-androsten-3 $\beta$ -ol-17-one
DHEA-S	dehydroepiandrosterone sulphate
DHT	dehydrotestosterone
DMEM	Dulbecco's modified eagles medium
<i>Escherichia coli</i>	<i>E. coli</i>
FAD	flavinadenine dinucleotide
fdx/fdr	ferredoxin/ferredoxin reductase
FMN	flavinmononucleotide
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
GR	glucocorticoid receptor
HDL	high density lipoprotein
HPA-axis	hypothalamic-pituitary-adrenal axis
HPG-axis	hypothalamic-pituitary-gonadal axis

HPLC	high performance liquid chromatography
HRT	hormonal replacement therapy
HSD	hydroxysteroid dehydrogenase
HSL	hormone sensitive lipase
IMM	inner mitochondrial membrane
LDL	low density lipoprotein
LH	luteinizing hormone
MR	mineralocorticoid receptor
OMM	outer mitochondrial membrane
P450	cytochrome P450
P450scc	cytochrome P450 cholesterol side-chain cleavage
pdb-id	protein data bank identification
PKA	cAMP-dependent protein kinase
PR	progesterone receptor
preg	pregnenolone
prog	progesterone
PSA	prostate-specific antigen
RODH	17 $\beta$ -hydroxysteroid dehydrogenase type 6
SHBG	sex hormone binding globulin
SNP	single nucleotide polymorphism
SRB1	scavenger receptor B1
SREBPs	sterol response binding proteins
SSCP	single strand conformational polymorphism
StAR	steroid acute regulatory protein
UPLC-MS	ultra performance liquid chromatography mass spectrophotometry

## CHAPTER 1

### INTRODUCTION

The study and understanding of steroid biosynthesis is pertinent to areas of physiology, medicine, and pharmacology. Investigating disorders confined to rare genetic lesions and the contribution of altered steroidogenic regulation in common clinical disorders have contributed greatly towards our present knowledge of steroid hormone biosynthesis. Major developments have led to a comprehensive understanding of steroid biosynthesis — the cloning of the steroidogenic acute regulatory protein (StAR) and ensuing studies pertaining to the intracellular transport of cholesterol; the expanding anthology of cytochrome P450s (P450) hydroxysteroid dehydrogenases (HSD); the increasing roles of electron transfer proteins and other cofactors associated with disease; and the illumination of additional steroidogenic pathways in classical and extra-glandular steroidogenic tissues have added substantial intricacies to the understanding of molecular steroidogenesis.

Steroid hormones are produced from the metabolism of cholesterol. Their structures are, therefore, directly related and all contain the characteristic cyclopentanophenanthrene or gonane 4-ring structure present in cholesterol (Miller and Auchus, 2011). Steroid hormones are important in that they regulate numerous developmental and physiological progression from fetal life through to adulthood. Glucocorticoids and mineralocorticoids are secreted by the adrenal glands and are vital in carbohydrate metabolism and the perpetuation of blood volume and the regulation of sodium levels, respectively. Androgens are mandatory for gametogenesis, secondary sexual characteristics and reproduction and are generally synthesized by the testes and ovaries. Adrenal glands do secrete the weaker androgens, dehydroepiandrosterone (DHEA), DHEA-sulphate (DHEA-S) and androstenedione (A4) but neither DHEA nor DHEA-S can activate the androgen receptor (AR). In peripheral tissues, C19 weak androgens serve as metabolic precursors for the synthesis of the potent C19 androgens, testosterone and dihydrotestosterone (DHT).

The adrenal C19 steroids are catalysed by cytochrome P450 17 $\alpha$ -hydroxylase/17,20 lyase (CYP17A1), an enzyme which functions at a key branch point in steroidogenesis with its two core activities determining which class of steroid hormones a given cell may produce. CYP17A1 catalyses the 17 $\alpha$ -hydroxylation of both pregnenolone (preg) and progesterone (prog), as well as C17-C20 cleavage of their 17 $\alpha$ -hydroxy derivatives 17 $\alpha$ -hydroxypregnenolone (17OHpreg) and 17 $\alpha$ -hydroxyprogesterone



(17OHprog) respectively, in a common active site. In the absence of CYP17A1 activities, only prog, mineralocorticoids (11-deoxycorticosterone and aldosterone) and the weak glucocorticoid, corticosterone, can be formed. The 17 $\alpha$ -hydroxylase activity is required for formation of the glucocorticoid cortisol and both 17 $\alpha$ -hydroxylase and 17,20-lyase activities are needed for C19 steroid production (in adrenal or gonads). Cytochrome P450 oxidoreductase (CPR) is the classical redox partner of CYP17A1 and other microsomal P450s and relies on the assistance of an electron transfer flavoprotein to shuttle electrons between reduced NADPH and the P450s heme center. In the adrenal glands, CYP17A1 17,20-lyase activity is reliant on CPR to facilitate the reaction (Yanagibashi and Hall, 1986). Furthermore, there is substantial evidence supporting the understanding that the interaction of CYP17A1 with cytochrome b5 (CYB5) profoundly increases the rate of 17,20-lyase activity, approaching that of the 17 $\alpha$ -hydroxylase reaction, by facilitating the transfer of the second electron (Lee-Robichaud *et al.*, 1995a).

CYP17A1 protein from different species exhibits similar catalytic activities in the  $\Delta$ 5 and  $\Delta$ 4 pathways towards preg and prog, respectively. However, the kinetics of the 17,20-lyase reaction have been shown to be remarkably different for the two hydroxylated substrates. In some species, the secretion of C19-steroids is not restricted only to the gonads. Lower vertebrate animals (i.e. rat, hamster) do not express adrenal CYP17A1 and thus are incapable of synthesizing adrenal C19-steroids. The guinea-pig adrenal favours C19-steroid production from 17OHprog via the  $\Delta$ 4 pathway. Human adrenal CYP17A1 (as well as bovine, sheep, porcine and other primates) favours the  $\Delta$ 5 pathway with the 17,20-lyase activity towards 17OHpreg producing DHEA, which is subsequently converted to A4 by 3 $\beta$ HSD (Lee-Robichaud *et al.*, 1995a; Katagiri *et al.*, 1995; Auchus *et al.*, 1998). Porcine CYP17A1 is unique in the fact that although it leads to rapid DHEA production from 17OHpreg, when expressed in the adrenal, it is also highly capable of producing significant levels of A4 from 17OHprog. The lyase activities are amplified when porcine CYP17A1 is expressed in the presence of CYB5. However, together with hydroxylase and lyase catalytic activities, porcine CYP17A1 exhibits an additional species-dependent activity in the catalysis of C16-steroids (androstenes) when expressed in the presence of elevated levels of CYB5, which is characteristic of the porcine testes. It is due to the versatility of porcine CYP17A1s catalytic activity, along with the efficiency at forming both  $\Delta$ 5 and  $\Delta$ 4 steroid products, that makes this species enzyme a suitable model for the manipulation of the primary protein structure to investigate the enzyme. Since the enzyme already favours the  $\Delta$ 5 pathway and DHEA formation, the  $\Delta$ 4 pathway will be studied to determine the influence of CYB5 on enzyme activity.

Chapter 2 presents an overview of steroidogenesis in which the catalytic roles of the P450 enzymes, together with the HSDs, with respect to the biosynthesis of adrenal steroid hormones, are discussed. The dual hydroxylase and lyase activities of CYP17A1 and the influence of CYB5 on the modulation of the lyase activity in the biosynthesis of adrenal androgens will be presented.

Chapter 3 and Chapter 4 describe the study into the catalytic activity of porcine CYP17A1 in which the following aims are addressed:

- to investigate the effect of the amino acid residues at positions 40 and 407 on the catalytic activity of porcine CYP17A1
- to clone porcine CYB5 from porcine liver tissue
- to investigate the effect of porcine CYB5 on the catalytic activity of porcine CYP17A1
- to compare the influence of porcine CYB5 to that of human, rat and angora CYB5 on the lyase activity of porcine CYP17A1

The investigation into the influence of mutations, at amino acid residue positions 40 and 407 in porcine CYP17A1, on the catalytic activity of the enzyme is described in Chapter 3. The amino acid sequence of porcine wild-type (wt) CYP17A1 cDNA (wt pCYP17A1), available in our laboratory, differed from the published amino acid sequences for CYP17A1 protein previously isolated from the adrenal (Chung *et al.*, 1987) and translated from the testes mRNA (Conley *et al.*, 1992). Sequence analysis of porcine wt CYP17A1 identified Val40 and His407 (designated -VH) amino acid residues, while the adrenal protein has Leu40 and His407 (designated -LH) residues and the testicular protein has Leu40 and Leu407 (designated -LL) residues.

Mutant constructs were thus prepared, using porcine wt CYP17A1 (Val40 and His407) to incorporate residues Leu40 and His407 (pCYP17A1-LH), as well as residues Leu40 and Leu407 (pCYP17A1-LL), to represent both the porcine adrenal and the testicular proteins, respectively. A single CYP17A1 gene is expressed in both human adrenal and testes and an amino acid sequence comparison of the translated protein with porcine CYP17A1 shows that human CYP17A1 exhibits Leu40 and His407 residues. Furthermore, human CYP17A1 has an Ala105 residue, which is associated with the 16 $\alpha$ -hydroxylase activity (Swart *et al.*, 2010). Porcine wt CYP17A1, as well as the adrenal and testes protein sequences, have a Leu residue at position 105. As porcine CYP17A1 catalyses low levels of 16 $\alpha$ -hydroxyprogesterone (16OHprog), the mutation Leu105Ala was also constructed (pCYP17A1-LA) to investigate the 16 $\alpha$ -hydroxylase activity. Constructs were transiently expressed in a non-steroidogenic mammalian (COS-1) cell line and the activities of wt and mutated constructs were investigated.

Chapter 4 presents an investigation into the influence of porcine, human, rat and angora CYB5, which all share a high degree of amino acid sequence identity, on the catalytic activity of porcine CYP17A1. Data, which will be presented and discussed, include the cloning of porcine cDNA encoding CYB5 by RT-PCR of total RNA isolated from porcine liver. CYB5 constructs were co-expressed in COS-1 cells with porcine adrenal CYP17A1 (wt pCYP17A1-VH) and pCYP17A1-LL (mutant construct representing testicular protein) and the influence of CYB5 on the conversion of prog was assayed to determine the influence of the different species of CYB5s on the catalytic activity of porcine CYP17A1.

Chapter 5 concludes the thesis with an overview of the results obtained in this study. Inferences pertaining to the structure/function relationship of porcine CYP17A1, without and in the presence of CYB5, will be presented.

## CHAPTER 2

### STEROID BIOSYNTHESIS AND THE ROLE OF CYP17A1 AND CYB5

#### 2.1 The endocrine system

The endocrine system is one of the body's two main communications systems, the nervous system being the second. Communication within the nervous system is expeditious, while the signals sent by the endocrine system may be delayed and last for longer periods of time. The endocrine system consists of glands that are not anatomically connected. These ductless glands secrete hormones, chemical messengers, which enter the blood stream and are carried away from the endocrine gland to the target cells upon which they purposefully act. A single gland may secrete multiple hormones due to different types of endocrine cells in the same gland or single cell may excrete more than one hormone (Widmaier *et al.*, 2004).

From a chemical standpoint, hormones fall into three chemical classes. *Amines* include the thyroid hormones, including follicle stimulating hormone (FSH) and leutenizing hormone (LH), the catecholamines: epinephrine and norepinephrine, which are produced by the adrenal medulla and dopamine, which is produced by the hypothalamus. There are a wide range of *peptide* hormones, including gonadotropin releasing hormone (GnRH) and adrenocorticotrophic hormone (ACTH), which are all synthesized by specific endocrine glands, including the gastrointestinal tract, heart, kidneys, liver and pancreas. Peptide hormones are synthesised within endocrine cells by the ribosomes, rough ER and Golgi apparatus, where they are finally stored in secretory vesicles in the active hormone form, waiting for cell stimulation. A single endocrine cell may synthesize, store and secrete multiple peptide hormones that differ in their effects on target cells. The classical *steroid*-producing endocrine glands are the adrenal glands (adrenal cortex), gonads (ovary and testis), placenta (during pregnancy) and the brain. All steroid hormones are synthesized from cholesterol. Unlike the hydrophilic amine and peptide hormones, upon synthesis, the lipid-soluble steroid hormones diffuse across the plasma membrane, enter the interstitial fluid and then enter the blood (Widmaier *et al.*, 2004).

## 2.2 The adrenal gland: a historical perspective on its function

A recent publication provides an informative historical overview of the scientists involved in the first discoveries of the adrenal gland as an endocrine gland, as well as the earliest documentations of steroids (Miller, 2013).

In 1563, Professor Eustachius was the first to correctly describe the anatomy of the adrenal, but due to political and religious sequestration his work was only published in 1714. In 1805, Cuvier distinguished the cortex and the medulla. Brown-Sequard later speculated that the adrenals secreted the “life-sustaining factors” referred to as hormones. He proved that a gland was indispensable for life and was the first who attempted hormonal replacement therapy (HRT). Many diseases associated with adrenal malfunction were identified and documented during the 1700s and 1800s. In 1912, Glynn correctly concluded that the adrenal medulla and cortex developed differently, were functionally different to each other and distinguished between sexual characteristics and blood pressure. Further advancements in understanding the adrenal and adrenocortical hormones were established with respect to symptoms of Addison and Cushing disease. In 1946, Seyle studied the stress response and conceptualized the hypothalamic-pituitary-adrenal axis (HPA-axis) of control and coined the terms ‘mineralocorticoid’ and ‘glucocorticoid’. In 1927, Wieland and Wiedaus set the baseline for identifying the molecular structure of cholesterol. However, the correct cyclopentanophenanthrene structure (see Figure 2-1) was resolved by Rosenheim and King in 1932 and thereafter estrone, androsterone and prog were isolated. This set the stage for studies on adrenal steroids. Amongst other researchers, Kendall, Reichstein and Hench contributed in the identification and isolation of 29 distinct steroids, including cortisone, corticosterone and cortisol. In 1950, they received the only Nobel Prize, ever awarded, for work pertaining to adrenal steroid hormones, which led to the administration of cortisone as a “miracle cure” for severely arthritic patients (Hench *et al.*, 1949; Miller, 2013).

The 1930s yielded fundamental characterisations as well as identified the precursor/product relationships, which ultimately led to the general understanding of steroidogenesis (Kendall *et al.*, 1934). Early studies from the 1950s revealed that steroid 21-hydroxylation by bovine adrenal microsomes was inhibited in the presence of carbon monoxide and was reversible by light, which led to the most important breakthrough: that steroid 21-hydroxylation was P450-mediated (Ryan and Engel, 1956; Miller, 2013). The following years revealed key discoveries concerning steroidogenic reactions being associated with P450 enzymes. The cloning of the cDNA encoding key steroidogenic enzymes

from a variety of animal sources proposed that there were less steroidogenic enzymes than there were steroidogenic reactions (Miller, 1988; 2013). It was concluded that generally, in different tissues, the same enzyme would catalyze one particular steroidogenic reaction.

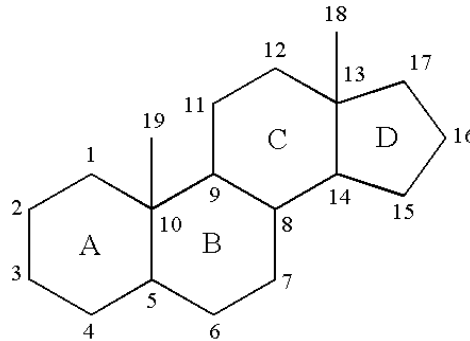


Figure 2-1: Cyclopentanophenanthrene 4-ring structure is common to all steroids. According to the standard convention of nomenclature, *numbers* indicate carbon atoms and *letters* designate rings. Methyl groups may be present at C10 and C13, hydroxyl groups may be present at C17, C16 or C3 and an alkyl side chain may be present at C17. The major C21 steroid precursors to steroid hormone biosynthesis, derived from cholesterol with an 8-C side-chain at C17, are preg (double bond between C5-C6) and prog (double bond between C4-C5), both possessing a 2-C side-chain at C17. 3 $\beta$ HSD isomerase activity is responsible for shifting double bonds between rings B and A. Steroids derived from preg follow the  $\Delta$ 5 pathway and possess C5-C6 double bonds and steroids derived from prog follow the  $\Delta$ 4 pathway and generally possess C4-C5 double bonds. For steroids with this basic structure, functional groups and hydrogen molecules are labelled as either  $\alpha$  or  $\beta$ , according to whether they are situated behind or in front of the plane of the page, respectively (reproduced from Moss, 1989).

## 2.3 Steroid hormones

Steroids are essential for vertebrate physiology during pre- and postnatal life. They are required for normal reproductive function and body homeostasis. In humans, there are five central groups of steroid hormones: progestagens (prog), androgens (testosterone), estrogens (estradiol), glucocorticoids (cortisol/corticosterone) and mineralocorticoids (aldosterone). Testosterone, and its potent derivative DHT, prog and estradiol are together referred to as the sex steroids while cortisol, corticosterone and aldosterone are collectively referred to as corticosteroids (Widmaier *et al.*, 2004). Figure 2-2 shows the metabolic pathway, which produces the three classes of steroid hormones, namely sex steroids and the mineralo-and glucocorticosteroids.

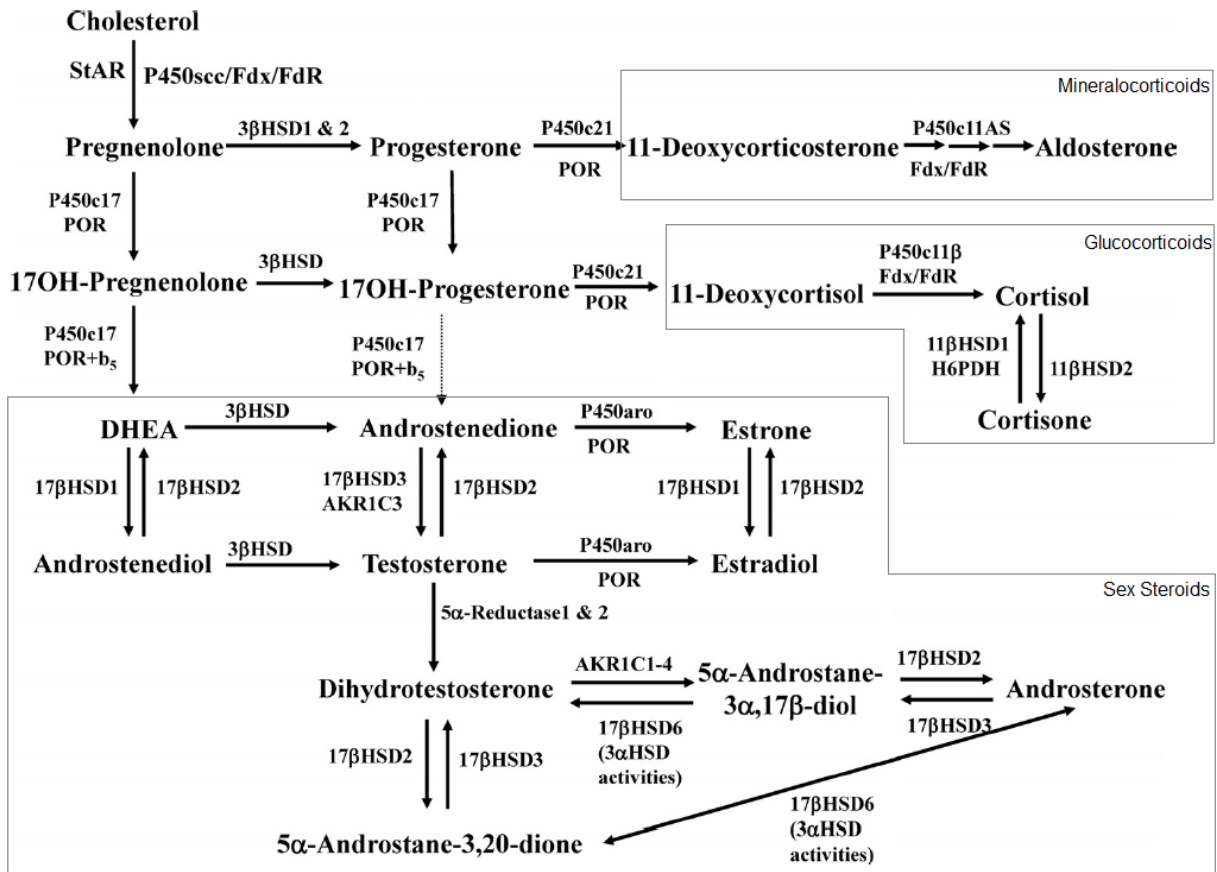


Figure 2-2: Overview of the biosynthesis of mineralocorticoids, glucocorticoids, estrogens and androgens in the adrenals, testes and ovaries. The steroidogenic enzymes, P450 and HSD, are shown at the reactions, which they catalyse, as well as the electron transfer proteins involved in the reaction: CPR (or POR) and ferredoxin/ferredoxin reductase (fdx/fdr). Cofactor CYB5 (or  $b_5$ ) may amplify specific enzyme activities. The formation of the weaker androgens, DHEA and A4, may take place in adrenals or not at all. Levels of androgens produced by adrenal glands are highly species-specific and dependent on the 17,20-lyase activity of CYP17A1. Complete conversion of androgens to estrone and estradiol take place only in the ovaries (reproduced from Miller and Auchus, 2011).

Although present during other phases of the estrous and menstrual cycles, progestagens are defined by their ability to maintain pregnancy. Synthetic progestagens (or progestins) are used as HRTs and in contraception (Voet and Voet, 2004). The progestagens (prog, 17OHprog and 17OHpreg) are the major precursors to the mineralocorticoids (prog), glucocorticoids (17OHprog), estrogens and androgens (17OHprog or 17OHpreg) (Miller and Auchus, 2011). Progestagens have anti-gonadotropic effects and at adequate concentrations inhibit the formation of prog and other sex steroids (Raudrant and Rabe, 2003). To avoid sex hormone levels from increasing beyond normal concentrations, the presence of prog inhibits the secretion of FSH and LH by negative feedback on the hypothalamic-pituitary-gonadal axis (HPG-axis), which is triggered by prog binding to and activating the prog receptor (PR). All steroidogenic tissues are in general capable of producing progestagens (Miller and Auchus, 2011). In

certain tissues, enzymes required for the formation of the final steroid product are not all found in a single cell. Theca cells, in the ovarian follicles, convert cholesterol to the androgen A4, which is subsequently converted to estrogen in the granulosa cells. The sex steroids are crucial for sexual development, sexual behaviour and reproduction and bind to their respective steroid receptors, AR and estrogen receptor (ER), in target tissues (Couse and Korach, 1998). The major postnatal production of these steroids commences at the onset of puberty and declines after menopause in females.

Glucocorticoids perform essential roles in regulating glucose metabolism. They are synthesised in the adrenal cortex and bind to the glucocorticoid receptor (GR) in target cells (Miller and Auchus, 2011). Glucocorticoids form part of the feedback mechanism in the immune system, which reduces inflammation (Rhen and Cidlowski, 2005). Thus, in medicine, they are utilised for properties assisting in the treatment of diseases that are associated with an overactive immune system, including asthma, allergies and autoimmune diseases. They also have inhibitory effects on lymphocyte proliferation and thus are involved in the treatment of lymphomas and leukemias (Pazirandeh *et al.*, 2002). Cortisol is the most important glucocorticoid in humans and regulates or assists a variety of important cardiovascular, metabolic, immunologic and homeostatic systems (Chrousos, 2009). Cortisol is synthesised from corticosterone (the inactive form) and its synthesis is triggered in response to stress or when blood glucocorticoid levels are low. Corticosterone also possesses glucocorticoid activity and is the predominant glucocorticoid in rodents.

Aldosterone binds to the mineralocorticoid receptor (MR) and its principle effects include the reabsorption of water and the induction and expression of epithelial sodium channels and the sodium/potassium ATPase in the distal convoluted tube and collecting duct of the nephron, hence regulating sodium reabsorption and potassium reuptake. Aldosterone action is a key determinant in blood pressure (Williams and Williams, 2003). Other steroids are also present in steroidogenic organs and in the circulation. These are referred to as steroid precursors as they need to be converted into the active steroid hormones in their target tissues. Table 2-1 shows a summary of the major steroids, site of production, trophic hormone responsible for regulation and their physiological functions.



Table 2-1: Major steroid hormones and their physiological functions (Hu *et al.*, 2010).

Steroidogenic Tissues	Trophic Hormone	Steroids(s)	Physiological Functions
<b>Ovary</b>			
Granulosa cells	FSH	Estradiol	Estrogen, a principal female sex steroid, required for growth and ovulation, responsible for secondary female sex characteristics, regulator of cardiovascular physiology, bone integrity and neuronal growth
Luteinized Granulosa/ luteal Cells	LH	Progesterone	A progestin, required for follicular growth and ovulation, responsible for changes associated with luteal phase of the menstrual cycle, essential for the establishment and maintenance of early pregnancy
Theca-interstitial Cells	LH	Testosterone Androstenedione	Androgens, precursors for estrogens, transported into granulosa cells, where they are converted into estradiol and other estrogens by aromatase (CYP19A1) enzyme
<b>Testis</b>			
Leydig cells	LH	Testosterone	The most prevalent male sex hormone (androgen); testosterone and its biologically active form, dihydrotestosterone (DHT) are necessary for normal spermatogenesis and development, responsible for secondary sex characteristics, responsible for increased muscle mass, sexual function, body hair and decreased risk of osteoporosis
<b>Adrenal gland</b>			
Z. glomerulosa Cells	ACTH, K <sup>+</sup> Angiotensin II	Aldosterone	The principal mineralocorticoid, raises blood pressure and fluid volume, enhances sodium reabsorption in the kidney, sweat gland, stomach and salivary gland and also enhances excretion of potassium and hydrogen ions from the kidney.
Z. glomerulosa Cells	ACTH	Cortisol	The dominant glucocorticoid in humans (in rodents, the major glucocorticoid is corticosterone), elevates blood pressure and Na <sup>+</sup> uptake, involved in stress adaptation, regulates carbohydrate, protein and lipid metabolism nearly opposite to that of insulin, influences inflammatory reactions and numerous effects on the immune system.
Z. reticularis Cells	ACTH POC-derived peptide Other factors	Androstenedione DHEA DHEA-sulfate	The function of adrenal androgens is not well understood, except that they contribute to the maintenance of secondary sex characteristics, may also be involved in the regulation of bone mineral density, muscle mass and may beneficial actions against type 2 diabetes and obesity
<b>Placenta</b>			
	Peptide growth Factors, cAMP	Progesterone Estrogens	Maintenance of pregnancy
<b>Brain</b>			
Neurons, Glial cells Purkinje cells	Neurotransmitters Neuropeptides	Progesterone Estradiol, DHEA, ALLO, THDOC	Neurosteroids are implicated in various processes such as proliferation, differentiation, activity and survival of nerve cells and a variety of neuronal functions including control and behavior, neuroendocrine and metabolic processes.

Steroid hormones are synthesised from cholesterol via a common precursor steroid, preg, which is the product of a single reaction catalysed by the first rate-limiting P450 (see Figure 2-2). All steroids possess the gonane skeleton (cyclopentanophenanthrene) or a skeleton derived there from. The basic structure has 17 carbons that consists of three 6-C rings to which a 5-C ring is attached, see Figure 2-1 (Moss, 1989). Carbons 10 and 13 both have attached methyl groups. Each carbon has one or two hydrogens attached.

Steroids are divided into groups according to the parent compound and the number of carbons that they possess. Cholestane (cholesterol) has 27 carbons, including the 17-C gonane structure. Pregnanes

contain 21 carbons and are referred to as C21 steroids, these include preg, prog and the corticosteroids. Androstanes have 19 carbons and include the androgens (C19 steroids). Estranes consist of 18 carbons and include the estrogens (C18 steroids). Steroid compounds differ in characteristics because of the occurrence of different functional groups attached to the basic steroid structure. Ketones and hydroxyl groups, double bonds, methyl- or ethyl groups attached to the four rings and the oxidation state of the carbon atoms in the rings, are typical functional groups common to all steroids (Moss, 1989). Preg is the first steroid produced: an essential intermediate in steroid synthesis and is therefore the cornerstone for synthesis of each class of steroid hormone. The molecular structure of preg is shown in Figure 2-3, as well as various active steroid hormones.

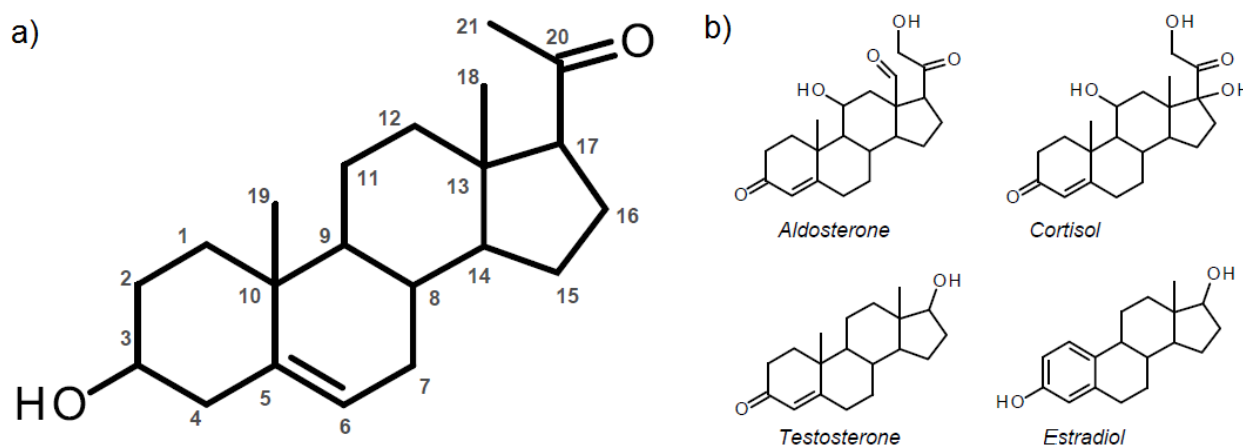


Figure 2-3: (a) Molecular structure of preg, a common steroid precursor. *Numbers* refer to carbon atoms in the steroid molecule and the nomenclature of the steroidogenic reactions and associated enzymes are derived from the number of the affected carbon atom. (b) Molecular structure of active steroid hormones: the mineralocorticoid aldosterone (C21), the glucocorticoid cortisol (C21), the androgen testosterone (C19) and the estrogen estradiol (C18).

The multitude of biochemical steps which occur during steroid synthesis from cholesterol each incorporate small molecular changes to each formed steroid and are exquisitely mediated by specific enzymes. The steroids produced by a particular cell therefore depend on the types and concentrations of enzymes present.

## 2.4 Cholesterol synthesis and delivery to steroidogenic tissue

Steroidogenic cells have the potential to acquire cholesterol from multiple sources. Cholesterol synthesis can be achieved *de novo* from acetate in the ER of all steroidogenic cells (Mason and Rainey,

1987). Furthermore, using receptor-mediated uptake methods, intracellular cholesterol can be acquired from plasma low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Gwynn and Strauss, 1982). Even though all major steroidogenic organs are capable of synthesizing cholesterol *de novo*; the adrenal and ovaries preferentially utilize cholesterol supplied by LDL and HDL, while testicular Leydig cells rely essentially on endogenously synthesized cholesterol. In contrast to human adrenals preferring LDL-derived cholesterol, rodent adrenals prefer synthesis from HDL via a receptor termed scavenger receptor B1 (SRB1) (Azhar *et al.*, 2003).

Intracellular cholesterol levels are controlled by a collection of transcription factors referred to as sterol response binding proteins (SREBPs). These proteins regulate the expression of genes, which are implicated in the biosynthesis of cholesterol, as well as fatty acids (Horton *et al.*, 2002). ACTH stimulates LDL receptors, which causes the uptake of LDL cholesterol, as well as stimulating the rate limiting enzyme of cholesterol synthesis: 3-hydroxy-methylglutaryl co-enzyme A reductase. Adequate concentrations of LDL suppress this enzyme. After receptor-mediated endocytosis, intracellular LDL cholesterol esters are either stored in endosomes or converted to free cholesterol, which are used in steroid hormone synthesis (Brown *et al.*, 1979). Figure 2-3 shows the main features of cholesterol delivery intracellular and into the mitochondria.

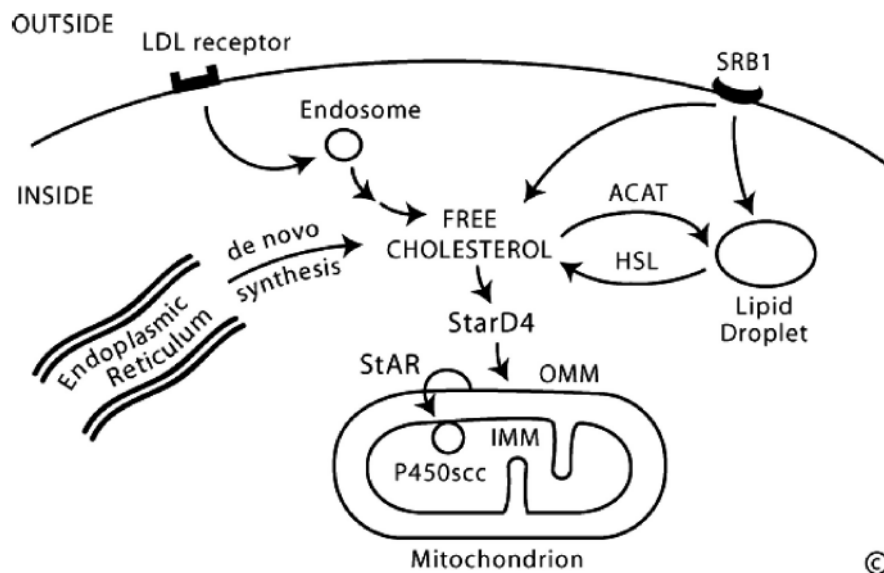


Figure 2-3: Cellular cholesterol economy and delivery to the mitochondria for steroid hormone synthesis (Miller and Auchus, 2011).

Enzymes of the acyl-coenzyme A: cholesterol acyltransferase (ACAT) family can esterify free cholesterol, which is stored as cholesterol esters in lipid vesicles. Furthermore, activation of hormone-sensitive lipase (HSL) allows access to this free cholesterol. Finally, free cholesterol can be bound by StarD4 for transport through the cytosol to target membranes, including the outer mitochondrial membrane (OMM) (Soccio and Breslow, 2003). However, StarD4 mediated transcytoplasmic transport is not the only means by which free cholesterol or cholesterol from intracellular lipid droplets can reach the OMM. Movement and fusion of adjacent cholesterol containing vesicles (Soccio and Breslow, 2004) as well as nonspecific bindings with sterol-carrier proteins (Gallegos *et al.*, 2001) can transport the nearly insoluble free cholesterol to the OMM. StAR protein is responsible for the swift translocation of cholesterol from the OMM to the inner mitochondrial membrane (IMM) (Miller, 2007a). Transport of cholesterol into the IMM initiates the start of steroidogenesis with the first catalytic reaction, mediated by P450<sub>scc</sub> and the production of the first C21 steroid precursor, preg.

## **2.5 Steroid hormone biosynthesis by endocrine tissues**

Steroidogenesis in the adrenal, ovaries and testes is predominantly under the regulation and control by tissue-specific trophic hormones; however the availability of sufficient levels of cholesterol, as a substrate, is a key determining factor in optimal production of steroid hormones. Only the adrenals can synthesize corticosteroids. The ovaries and testes, but not the adrenals, can synthesize estrogens. The adrenals, ovaries and testes can all synthesize progestagens and androgens though the amounts of androgens produced by the adrenals depend greatly on the species and the concentration and expression of steroidogenic enzymes (Miller and Auchus, 2011).

### **2.5.1 The biosynthesis pathway**

The comprehensive metabolic pathway for the biosynthesis of active steroid hormones from cholesterol commences with the mediated transport of cholesterol into the mitochondria of a steroidogenic cell. A side chain cleavage reaction yields the steroid precursor, preg, which is systematically and enzymatically transformed via a cascade of reactions to produce biologically active steroid hormones (see Figure 2-2).

The collection of enzymes controlling each reaction in the pathway, are referred to as steroidogenic enzymes and exist in two distinct groups of proteins: P450s and HSDs. Reactions, which are mediated by P450 enzymes, are irreversible with regards to mechanism and physiology (Hall, 1986). HSD-mediated reaction mechanisms are reversible and can proceed bi-directionally, although each HSD contributes to steroid flux by either oxidative or reductive mechanisms *in vivo* (Goosen *et al.*, 2012).

Steroidogenic enzymes are only expressed in certain cell types. This cell-specific level of expression determines the capabilities of each cell to synthesize different steroid hormones. Table 2-2 shows a summary of the sites of steroid enzyme expression in steroidogenic tissues and the major steroid hormones produced.

Table 2-2: Specific expression of steroidogenic enzymes in various cell types in the adrenal cortex, ovary and testes (Hanukoglu, 1992).

	Adrenal cortex			Ovary				Testis
	Zona glomerulosa	Zona fasciculata *	Theca interna	Granulosa (preantral follicle)	Granulosa (preovulatory follicle)	Luteinized theca (corpus luteum)	Luteinized granulosa (corpus luteum)	Leydig (interstitial tissue)
P450scc	+	+	+	-	+	++ <sup>a</sup>	++	+
3 $\beta$ -HSD	+	+	+	+	+	++	++	+
CYP11B1	-	+	-	-	-	-	-	-
CYP11B2 **	+	-	-	-	-	-	-	-
CYP18	+	-	-	-	-	-	-	-
CYP17	-	+ <sup>b</sup>	+	-	-	+	-	+
CYP21	+	+	-	-	-	-	-	-
CYP19 ***	-	-	-	-	+	-	+ <sup>c</sup>	-
17 $\beta$ -HSD	?	+	+	-	-	-	-	+
<b>Major steroid product</b>	<b>Mineralo-corticoid</b>	<b>Glucocorticoid Androgen</b>	<b>Androgen</b>	<b>Estrogen</b>	<b>Progestins Progesterone</b>	<b>Progesterone Androgen</b>	<b>Progesterone Estrogen</b>	<b>Androgen</b>

\* and zona reticularis

\*\* Aldosterone synthase

\*\*\* Aromatase

a ++, indicates much higher levels only relative to ovarian granulosa cells and not other cells

b Not expressed in rat, hamster and rabbit adrenals

c Expression dependent on specie

## 2.5.2 Cytochrome P450s

P450 steroid enzymes are all members of a protein superfamily represented in all kingdoms (bacteria, fungi, plants and mammals) with expression in the majority of species. They all contain a single heme group and are comprised of ~500 amino acids (Aguiar *et al.*, 2005). When P450s are complexed *in vitro* with exogenous carbon monoxide, and are reduced, they absorb light at 450 nm, hence the name P450 (pigment 450) (Nelson *et al.*, 1996). Over 3000 enzymes have been identified in nature and the human genome project has identified 57 human enzymes with critical physiological roles. The development of various diseases and disorders have been linked to P450s, including cancer and

cardiovascular- and endocrine dysfunction. Numerous levels of regulation contribute to maintaining the proper function of P450s, which include transcription, translation and posttranslational modifications of the proteins (Tee *et al.*, 2008).

Mammals express multiple enzymes concurrently in an array of tissue types, cell types and subcellular compartments (Guengerich, 2001). Mammalian P450s are all membrane bound. The first reported mammalian P450 structure offered insight into the comprehensive understanding of the flexible nature of P450s, which allows them to accommodate a range of substrates with different shapes and sizes (Williams *et al.*, 2000a; Williams *et al.*, 2000b). This is particularly true of the xenobiotic P450 enzymes, which are involved in the metabolism of drugs and carcinogens, while a second group of P450 enzymes have high substrate specificity, catalysing the biosynthesis and metabolism of endogenous compounds, including cholesterol, steroids, vitamins and eicosanoids.

One of the most central discoveries that emerged from molecular studies is that almost every P450 enzyme protein is encoded by a single gene (Miller and Auchus, 2011). A second notable finding is that some enzymes catalyse more than one reaction in the pathway. In general, P450s are capable of hydroxylating substrates as well as cleaving carbon-carbon bonds within steroid substrates. All P450s function as monooxygenases. Their heme centres activate molecular oxygen and incorporate one hydroxyl group into substrates by reducing two atoms of dioxygen to one hydroxyl group and one water molecule, together with the concomitant oxidation of the electron donor NADPH.

There are two biochemical classifications of P450 enzymes and they are classified according to their intracellular locations and the mechanisms by which they acquire electrons from NADPH (Miller, 2005). Type 1 P450s are associated with the IMM (including certain bacterial systems), where they receive two electrons from the reduction of NADPH via an electron transfer chain of two proteins: *fdx*, a flavoprotein also referred to as adrenodoxin reductase (*adr*) and *fdx*, an iron-sulfur protein also referred to as adrenodoxin (*adx*). Type 2 P450s are present in the ER, where they receive electrons from NADPH via membrane-bound CPR and are sometimes assisted by CYB5 (Pandey and Flück, 2013). Figure 2-5 shows these electron transfer mechanisms.

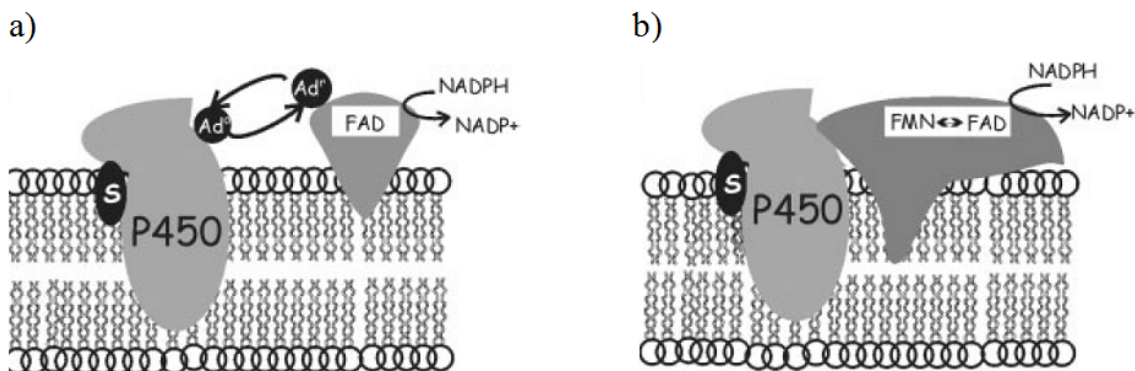


Figure 2-5: P450 electron transfer from NADPH. (a) In the mitochondrial system, two high potential electrons are transferred from NADPH to a flavoprotein *Adr* (or *fdr*) via its coenzyme flavin adenine dinucleotide (FAD). The electrons are shuttled one at a time by *Ad* (*adx* or *fdx*), a non-heme iron-sulfur protein, to the Type 1 P450 enzyme to hydroxylate or cleave the substrate (s). (b) The microsomal electron transfer system involves only one protein, CPR containing two flavin prosthetic groups: FAD and flavin mononucleotide (FMN). Electrons are transferred from NADPH, via the two flavin domains, to the type 2 P450 enzymes to modify the substrate (Payne and Hales, 2004).

### 2.5.2.1 Mitochondrial (type 1) P450 enzymes

Mitochondrial enzymes include cholesterol side-chain cleavage (P450<sub>scc</sub> or CYP11A1), P450 11 $\beta$ -hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2).

The predominant sites of P450<sub>scc</sub> expression are in the adrenal cortex (all three zones), ovary (theca and granulosa cells), testes (Leydig cells) and placenta (Ishimura and Fujita, 1997). P450<sub>scc</sub> catalyses the rate limiting step of steroid hormone biosynthesis. It converts cholesterol to preg via three distinct reactions: 22-hydroxylation, 20 $\alpha$ -hydroxylation, and scission of C20-C22 bond. Figure 2-6 shows these sequential reactions.

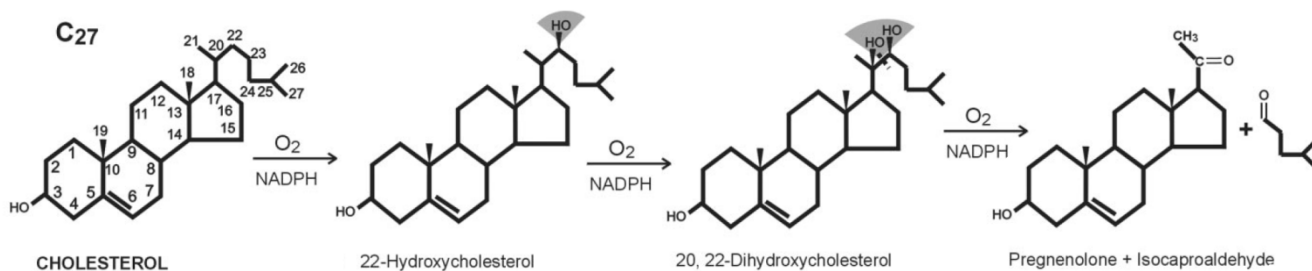


Figure 2-6: Three sequential reactions catalysed by P450<sub>scc</sub> in the formation of preg from cholesterol, via two hydroxylated cholesterol intermediates, utilizing one molecule of oxygen and one molecule NADPH per reaction (reproduced from Payne and Hales, 2004).



The rate limiting nature of this reaction is dependent on access of cholesterol (delivery and transport into the mitochondria) to the substrate-binding site of P450<sub>scc</sub> (Stocco, 2001). The hydroxylated intermediates are tightly bound to P450<sub>scc</sub>, whereas the dissociation constant of final product preg is 40 to 600-fold more than that of the hydroxylated intermediates (Orme-Johnson, 1990). Preg diffuses out of the mitochondria for further enzyme modifications by type 2 P450 enzymes.

CYP11B1 and B2 are located in the IMM. They are essential in final product formation of mineralocorticoids and glucocorticoids. Steroid products produced by type 2 enzymes diffuse back into the mitochondria for corticoid synthesis. CYP11B1 catalyses 11 $\beta$ -hydroxylation of 11-deoxycorticosterone and 11-deoxycortisol yielding corticosterone and cortisol, respectively (Payne and Hales, 2004). CYP11B1 can also hydroxylate C18 of 11-deoxycorticosterone or corticosterone to form 18-hydroxycorticosterone, although it is incapable of catalysing the oxidation of the 18-hydroxy group to form aldosterone. CYP11B2, also known as aldosterone synthase, catalyses aldosterone synthesis from 11-deoxycorticosterone. CYP11B2 catalyses three sequential reactions, utilizing one molecule of oxygen and one molecule of NADPH per reaction and the mitochondrial electron transfer system. CYP11B1 and 2 expression is restricted to the adrenal cortex; B2 is solely expressed in the zona glomerulosa and B1 is expressed in the zona fasciculata and zona reticularis (Ishimura and Fujita, 1997).

#### 2.5.2.2 Microsomal (type 2) P450 enzymes

The P450 enzymes that target the ER include CYP17A1, P450 aromatase (CYP19) and P450 21-hydroxylase (CYP21A2).

CYP17A1 is expressed in all classical steroidogenic tissues, with some species-related differences in the adrenal glands, ovaries and placenta (Ishimura and Fujita, 1997). In the adrenal gland, CYP17A1 is only expressed in the zona reticularis and zona fasciculata, but not in the zona glomerulosa (Hyatt *et al.*, 1983). CYP17A1 is essential for either directing the  $\Delta$ 5- and  $\Delta$ 4 pathways towards glucocorticoid (cortisol) synthesis, via its 17 $\alpha$ -hydroxylase activity. Its subsequent 17,20-lyase activity results in the pathway producing the precursors for sex steroids synthesis.

Sites of CYP19 are abundant, which includes the ovaries, testes and placenta, however, levels of expression have species-dependent differences and more so during pregnancy (Simpson *et al.*, 1994). CYP19 catalyses the conversion of the C19 androgens (A4 and testosterone) to the C18 estrogens



(estrone and estradiol, respectively). The reaction involves CPR, with three molecules of oxygen and three molecules of NADPH involved in hydroxylation and peroxidation reactions, resulting in the signature benzene structure.

Steroid hormone synthesis may occur in peripheral tissues but not from cholesterol. Alternatively, they are produced as products of metabolism of circulating precursor steroids, previously formed in the endocrine glands. Adipose tissue is capable of the conversion of androgens to estrogens and the conversion of testosterone to the androgen derivative, DHT, occurs in the skin (Auchus, 2004). Adipose tissue has substantial aromatase activity and adequately transforms A4 to estrone and testosterone to estradiol, with less efficiency and serves as the principle mechanism of estrogen production in postmenopausal women (Simpson *et al.*, 2002).

Aromatase excess syndrome is as a result of a defunct *CYP19A1* gene, which causes the over-expression of aromatase and an increase in estrogen production leading to gynecomastia in males and gigantomastia in females (Kamat *et al.*, 2002). Aromatase deficiency may lead to the inhibition of estrogen production, resulting in virilization of a female at birth and in both sexes, the lack of epiphyseal closure resulting in tall stature of the individuals (Herrmann *et al.*, 2002).

CYP21A2 expression is localized to the adrenal cortex and is mandatory for the synthesis of gluco- and mineralocorticoids (Rice *et al.*, 1990). CYP21A2 is responsible for C21 hydroxylation of  $\Delta^4$  steroids, prog and 17OHprog, yielding 11-deoxycorticosterone and 11-deoxycortisol, respectively, as corticoid precursors (Payne and Hales, 2004). One molecule of oxygen and one molecule of NADPH are utilized in the reaction and catalysis takes place in the ER. As the adrenal zona glomerulosa cells do not express CYP17A1, prog cannot be typically 17 $\alpha$ -hydroxylated thus prog is the sole substrate for CYP21A2 in these cell types. Furthermore, because CYP11B2 is also expressed in these zona glomerulosa, CYP21A2 is essential for providing the steroid precursors for aldosterone synthesis.

A defect within the *CYP21A2* gene causes an interruption in the normal development of the enzyme, termed congenital adrenal hyperplasia (CAH) or 21-hydroxylase deficiency. Abnormal CYP21A2 activity reduces cortisol synthesis and a concomitant reduction in cortisol levels. Hyperplasia of the adrenal cortex results in the elevation of ACTH levels. ACTH initiates the transport of cholesterol into the mitochondria and the synthesis of preg. Steroid precursors prog, 17OHpreg and especially 17OHprog, accumulate in the adrenal cortex and in circulating blood. 21-Hydroxylase activity is not involved in the synthesis of androgens; however, 21-hydroxylase deficiency leads to a considerable

amount of 17OHpreg being diverted to the synthesis of DHEA, A4 and testosterone in the first trimester of fetal life in both sexes (Lee, 2001).

### 2.5.3 Hydroxysteroid dehydrogenases

HSDs are essential to steroid synthesis and include 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta$ 5- $\Delta$ 4-isomerases (3 $\beta$ HSD), 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ HSD) and 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD). They have a molecular mass of ~35-45 kDa and do not possess heme groups. In contrast to the single gene expression system for P450s, multiple isoforms exist for 3 $\beta$ HSD and multiple isozymes exist for 17 $\beta$ - and 11 $\beta$ HSD, which are all products of distinct genes (Bellemare *et al.*, 2005). The number of isoforms/isozymes differ among species with regards to tissue distribution, catalytic activity (dehydrogenase or reductase), substrate and cofactor specificity and subcellular localization (Miller and Auchus, 2011). All isoforms (except type 5 17 $\beta$ HSD) belong to the same phylogenetic protein family, namely the short-chain alcohol dehydrogenase reductase superfamily (Miller and Auchus, 2011). Type 5 17 $\beta$ HSD belongs to the aldo-ketosteroid reductase superfamily (Bellemare *et al.*, 2005). All HSDs are involved in the reduction and oxidation of steroid hormones.

#### 2.5.3.1 3 $\beta$ HSD

3 $\beta$ HSD isoforms exhibit a high degree of amino acid sequence identity and are classified according to two functional groups: dehydrogenase/isomerase or 3-ketosteroid reductase, the latter not taking part in active steroid hormone synthesis. 3 $\beta$ HSD/isomerases play a pivotal role in synthesising all classes of active steroids and are expressed in all steroidogenic tissues in a cell- and tissue-specific manner. Two distinct forms have been identified in humans, 3 $\beta$ HSD1 and 2 (Penning, 1997). Type 1 expression occurs in the placenta, skin and breast tissue and type 2 occurs in the adrenal gland, ovary and testis (Simard *et al.*, 1996). 3 $\beta$ HSD/isomerases are mitochondrial or microsomal membrane bound enzymes, depending on the type of cell in which they are expressed (Rheume *et al.*, 1991). They function at key branch points, converting progestagens and androgens of the  $\Delta$ 5 pathway to progestagens and androgens of the  $\Delta$ 4 pathway (see Figure 2-2) or more specifically, 3 $\beta$ -hydroxy-5-ene steroids into 3-keto-4-ene steroids through sequential dehydrogenase and isomerase reactions.

In the first dehydrogenation reaction of the steroid substrate, NAD<sup>+</sup> is reduced to NADH with the production of a  $\Delta$ 5-3 ketosteroid intermediate and together with NADH, remains in complex with the

protein. Bound NADH induces a conformational change within the enzyme. This results in the ensuing isomerisation of the adjacent carbon-carbon double bond from the  $\Delta 5$  position (between carbons 5 and 6) to the  $\Delta 4$  position (between carbons 4 and 5) of the bound steroid intermediate, ultimately yielding the corresponding  $\Delta 4$  steroid (Thomas *et al.*, 2003). Substrates are preg, 17OHpreg and DHEA and steroid products are prog, 17OHprog and A4, respectively. Figure 2-7 shows the two-step conversion of  $\Delta 5$  androgen 5-androsten-3 $\beta$ -ol-17-one (DHEA) via the  $\Delta 5$ -3 keto intermediate 5-androstene-3,17-dione to  $\Delta 4$  androgen 4-androstene-3,17-dione (A4).  $3\beta$ HSD is also capable of converting 5-androstene-3 $\beta$ ,17 $\beta$ -diol (androstenediol), a product of DHEA metabolism by  $17\beta$ HSD, to testosterone (Payne and Hales, 2004).

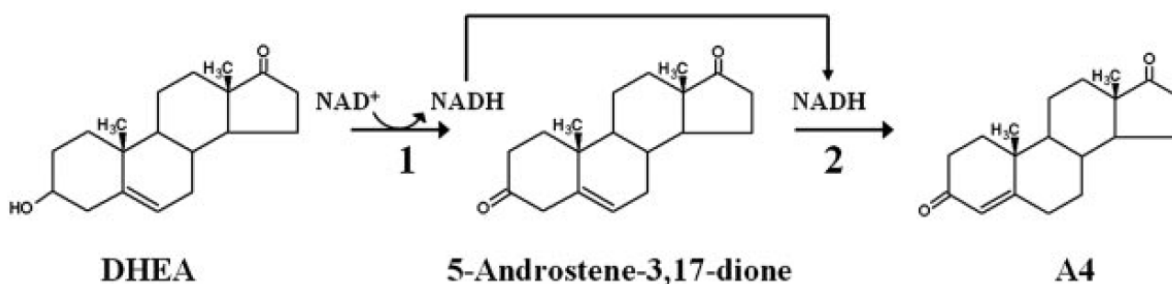


Figure 2-7:  $3\beta$ HSD-mediated conversion of a 3-hydroxy-5-ene steroid (DHEA) to a 3-keto-4-ene steroid (A4). This conversion involves (1) a dehydrogenation reaction via NAD<sup>+</sup> reduction and (2) a second isomeration reaction due to enzyme-bound NADH. The steroid intermediate formed is 5-androstene-3,17-dione (Thomas *et al.*, 2003).

Steroid  $5\alpha$ -reductases are usually grouped with HSDs however they reduce alkenes with single carbon-carbon bonds to their saturated state (Miller and Auchus, 2011). Testosterone is converted to DHT by two forms of steroid  $5\alpha$ -reductase (encoded by genes *SRD5A1* and *SRD5A2*).  $5\alpha$ -Reductase type 1 is found especially in liver and skin and type 2 predominates in urogenital tissues. In men, ~5% of testosterone undergoes  $5\alpha$ -reduction to form DHT. DHT has 2-3-fold greater affinity for ARs than testosterone and has 15-30-fold greater affinity than adrenal androgens. The dissociation rate of testosterone from the receptor is 5-fold faster than for DHT (Grino *et al.*, 1990). Idiopathic hirsutism occurs in hyperandrogenic women associated with increased  $5\alpha$ -reductase activity and the concomitant increase in levels of DHT (Azziz *et al.*, 2000).

### 2.5.3.2 17 $\beta$ HSD

17 $\beta$ HSDs, also known as 17-ketosteroid reductase, catalyze the final reaction in active gonadal steroid hormone biosynthesis, but are not involved in active hormone synthesis in the adrenal; however, they do produce precursors that are transported to the gonads. There are several different 17 $\beta$ HSD isoforms, functioning either as oxidases or reductases. They differ in substrate preferences and sites of expression. There are at least 14 human 17 $\beta$ HSD isoforms; these isoforms vary widely in size, structure, substrate specificity, cofactor utilization, subcellular localization and physiological functions (Peltoketo *et al.*, 1999). While the 3 $\beta$ HSDs exhibit a high degree of amino acid sequence identity the 17 $\beta$ HSD isozymes do not.

17 $\beta$ HSDs catalyze the reversible conversion of the inactive 17-keto (reduction) to the active 17 $\beta$ -hydroxy (oxidation) groups in androgens and estrogens (Payne and Hales, 2004). These reactions include the interconversions of A4 and testosterone; DHEA and androstenediol; estrone and estradiol; androsterone and 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (androstenediol), and 5 $\alpha$ -androstane-3,20-dione (androstanedione) and DHT. They use NADH/NADPH as the cofactor in the conversion of estrone to estradiol and A4 to testosterone (Luu-The, 2001; Peltoketo *et al.*, 1999). Figure 2-8 shows the activities of steroidogenic 17 $\beta$ HSD types 1 and 3 catalysing reactions in estradiol and testosterone formation, respectively.

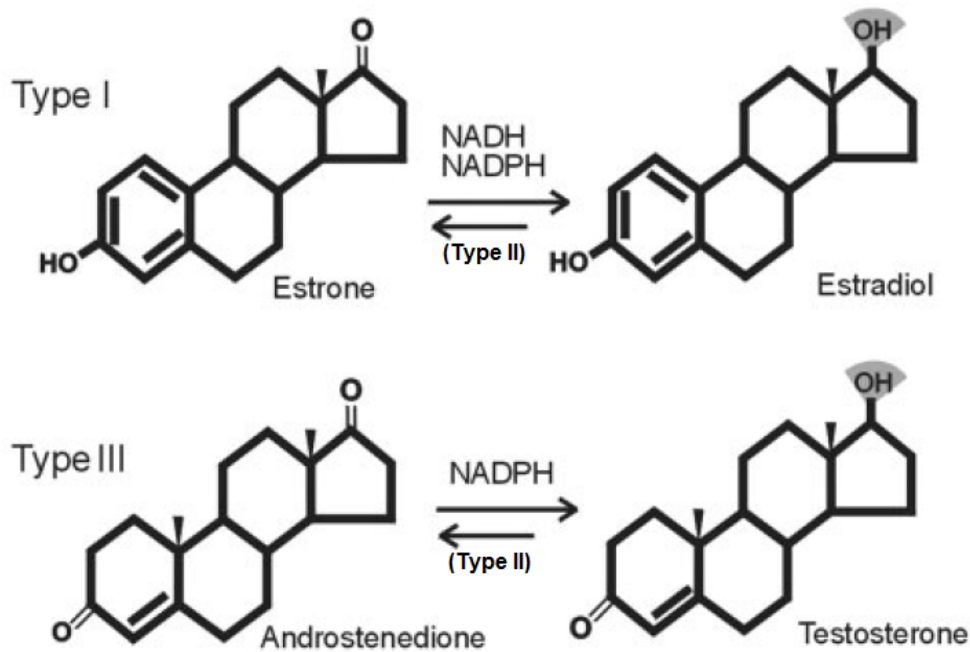


Figure 2-8: Enzymatic reactions catalyzed by 17βHSD isozymes. Human forms 1 and 3 act as reductases. Type 1 converts estrone to estradiol and type 3 converts A4 to testosterone with the reversible reactions catalysed by type 2 (Payne and Hales, 2004).

In humans, DHEA accounts for the majority of androgen secreted from the adrenal gland. 17βHSDs are expressed in several cell types, including the prostate epithelials. Type 1 is responsible for the conversion of DHEA to androstenediol, another significant precursor steroid in the formation of testosterone and or the estrogens (Miller and Auchus, 2011). Type 2 is responsible for the transformation of androstenediol back to DHEA. An important field of research is the influence of androgens on prostate and ovarian cancer. A study showed that orchiectomy reduces plasma concentration of testosterone by more than 90% but reduces plasma androstenediol by only 50% (Belanger *et al.*, 1986). Although androstenediol is a major metabolite of DHEA metabolism in human prostate homogenates (catalysed by prostate 17βHSDs), it has not yet been reported whether prostate cancer cells, postorchiectomy, are capable of forming androstenediol (Mitamura *et al.*, 2002). Mizokami *et al.* (2004) found that adrenal androstenediol is present in prostate cancer tissue even after androgen deprivation therapies (Mizokami *et al.*, 2004). Furthermore, androstenediol activates mutant ARs in cancer cells and is more efficient at activating these receptors than activation by DHT, inducing more cell proliferation, more expression of prostate-specific antigen (PSA) mRNA and more PSA promoter (Mizokami *et al.*, 2004). Plaza *et al.* (2010) reported increased conversion of DHEA to

androstenediol in endometria from untreated women with polycystic ovarian syndrome (Plaza *et al.*, 2010).

17 $\beta$ HSD type 2 converts testosterone to A4 but after 5 $\alpha$ -reduction of testosterone, type 2 is responsible for the conversion of DHT to androstanedione interconverted by 17 $\beta$ HSD type 6 (RODH) to androsterone. 17 $\beta$ HSD type 6 also exhibits modest 3-C isomerase activity between androstanediol and androsterone isomers ((Miller and Auchus, 2011). 17 $\beta$ HSD type 5 (AKR1C3) forms part of the aldo-keto reductase family and can also convert DHT to androstanediol, where 17 $\beta$ HSD type 2 converts it to androsterone. Androsterone is ultimately an inactive metabolite of testosterone breakdown, though it can be an effector on its own by being converted back to DHT via 17 $\beta$ HSD type 3 and 6. Table 2-3 shows the types of 17 $\beta$ HSD isoforms involved in steroidogenic reactions.

Table 2-3: Principle oxidase and reductase steroidogenic reactions catalysed by 17 $\beta$ HSDs (reproduced from Miller and Auchus, 2011).

17 $\beta$ HSD type	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6
Other names					AKR1C3	RODH
Preferred direction	Reduction	Oxidation	Reduction	Oxidation	Reduction	Oxidation
Favored cofactor in intact cells	NADPH	NAD <sup>+</sup>	NADPH	NAD <sup>+</sup>	NADPH	NAD <sup>+</sup>
Estrone $\rightarrow$ estradiol	Major		Minor			
Estradiol $\rightarrow$ estrone		Major		Trace		
16OH-estrone $\rightarrow$ estriol	Major					
Estriol $\rightarrow$ 16OH-estrone		Major		Trace		
DHEA $\rightarrow$ androstenediol	Modest		Modest			
Androstenedione $\rightarrow$ testosterone	Trace		Major		Minor	
Testosterone $\rightarrow$ androstenedione		Major		Trace		
DHT $\rightarrow$ 5 $\alpha$ -androstane-3,20dione		Major		Trace		Modest
5 $\alpha$ -Androstane-3,20dione $\rightarrow$ DHT			Major			
DHT $\rightarrow$ 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ diol					Modest	
5 $\alpha$ -Androstane-3 $\alpha$ ,17 $\beta$ diol $\rightarrow$ DHT						Modest
5 $\alpha$ -Androstane-3 $\alpha$ ,17 $\beta$ diol $\rightarrow$ androsterone		Modest				
Androsterone $\rightarrow$ 5 $\alpha$ -androstane 3,20dione						Modest

### 2.5.3.3 11 $\beta$ HSD

Although classical steroid hormones are classified as either gluco- or mineralocorticoids, the cloning and subsequent expression of MR demonstrated that these receptors had equivalent affinities for both aldosterone and cortisol (Arriza *et al.*, 1987). In mineralocorticoid-receptive tissues, cortisol is catalytically converted to a metabolically inactive steroid, cortisone (Funder *et al.*, 1988). The reversible reactions between the active and inactive forms are mediated by two 11 $\beta$ HSD isozymes (White *et al.*, 1997). 11 $\beta$ HSD type 1 (11 $\beta$ HSD1), a 34 kDa dimer, is expressed mostly in glucocorticoid-receptive tissues such as the liver, testis, lung, adipose tissue and proximal convoluted

tubule (Seckl *et al.*, 2004). Type 1 catalyzes the oxidation of cortisol to cortisone, which involves cofactor NADP<sup>+</sup>, and the reduction of cortisone to cortisol, which involves cofactor NADPH, with cortisone reduction being the dominant reaction. Catalysis is dependent on which cofactor is available; however, the enzyme can only function in the presence of elevated steroid substrate concentrations (Moore *et al.*, 1993). 11 $\beta$ HSD type 2 (11 $\beta$ HSD2) is a 41 kDa protein and only has 21% amino acid sequence identity with 11 $\beta$ HSD1, whereas 11 $\beta$ HSD2 and 17 $\beta$ HSD2 share 37% identity and both favour steroid oxidation *in vivo*. 11 $\beta$ HSD2 catalyzes only the oxidation of cortisol to cortisone using NAD<sup>+</sup>. 11 $\beta$ HSD2 expression occurs in mineralocorticoid-receptive tissues and therefore, serves to limit MR activation to the effects of “true” mineralocorticoids, aldosterone or deoxycorticosterone (Miller and Auchus, 2011).

#### 2.5.4 Regulation of steroid synthesis and enzyme expression

Chemical regulation of steroidogenesis is predominantly under the control of pituitary trophic hormones and the second rate-limiting step of steroid synthesis is the cleavage of cholesterol side chains by P450<sub>scc</sub>, during the first committed reaction in steroid biosynthesis. This reaction is stimulated in steroidogenic cells of the adrenal cortex by ACTH, which initiates glucocorticoid production, while LH stimulates steroidogenic cells in the ovary and testes, to release steroid hormones (Purves *et al.*, 2001).

The expression of P450<sub>scc</sub> proteins occurs in all steroidogenic cells and is regulated by the trophic hormonal system, which is cell-type specific (Hanukoglu, 1992; Guo *et al.*, 2003). In the zona fasciculata of adrenal cortex cells, the expression of mRNA encoding for P450<sub>scc</sub> is induced by ACTH (Hanukoglu *et al.*, 1990). ACTH is released into the bloodstream from the anterior pituitary, as a biological reaction to stress situations. Trophic hormones increase CYP11A1 gene expression through transcription factors (SF1) and by the  $\alpha$  isoform of activating protein 2 (AP2) (Lavoie and King, 2009).

P450<sub>scc</sub> is active at all times; however, its activity is first and foremost restricted by the supply of cholesterol from the OMM to the IMM. The supply of cholesterol is considered to be the true rate-limiting step in steroidogenesis and is facilitated predominantly by StAR or STARD1, a putative regulator and cholesterol-shuttling protein (Stocco, 2001). Within P450<sub>scc</sub> tertiary structure, the start domain contains the cholesterol-binding hydrophobic tunnel (Stocco, 2001).



In response to cellular stimulation for the synthesis of particular steroids, the amount of StAR available to transfer cholesterol to the IMM, confines the rate of the reaction (in acute regulation). With chronic or prolonged cell stimulation, the capacity of the system to synthesise the steroid becomes questionable. Congenital lipoid adrenal hyperplasia is a disease associated with mutations in the StAR gene, where steroid hormone biosynthesis is compromised (Hashemipour *et al.*, 2012).

Trophic hormone stimulation of any steroidogenic cell results in G-protein activation, which stimulate adenylate cyclase activity; elevates intracellular cAMP levels and the concomitant activation of protein kinase A (PKA). Activation of PKA signalling pathways is a mode of regulation acting either independently or synergistically with other forms of regulation. PKA action involves the phosphorylation of proteins (cholesterol ester hydrolase), as well as transcription factors and cAMP response-element binding proteins. The expression of StAR protein is regulated by the cAMP/PKA signalling cascade. Regulation of steroidogenesis can also be modulated through signal transduction pathways which do not require cAMP and or protein (StAR) synthesis. The PKA-independent signalling pathways, as well as the protein kinase C pathway, arachidonic acid metabolites, growth factors, chloride ions and the calcium messenger system, regulate or modulate steroid hormone biosynthesis (Stocco *et al.*, 2005).

Chronic regulation includes the over-transcription and translation of genetic sequences encoding for enzymes of the steroid biosynthesis pathways (Simpson and Waterman. 1988). A nuclear DNA-binding protein, termed SF-1 or Ad4BP, is a key nuclear component, which determines cell-specific expression of P450 and some HSD steroidogenic enzymes in the gonads and adrenals (Lala *et al.*, 1992; Morohashi *et al.*, 1992). This protein recognizes and binds variations of an AGGTCA sequence motif found in the proximal promoter. Other factors are also necessary for determining maximal expression, namely tissue-specific expression with cell-specific elements located within the enzymes gene sequence.

ACTH stimulates adrenal zona reticularis and -fasciculata cells; LH stimulates ovarian theca, corpus lutea and testicular Leydig cells and FSH stimulates ovarian granulosa cells. Peptide hormones perform via G protein-coupled receptors, which activate adenylate cyclase and increases cAMP levels. Increased cAMP levels initiates the synthesis of steroidogenic P450 and 3 $\beta$ HSD enzymes, specific to those cells. cAMP responsive sequences are located in the promoter sequence regions of genes encoding steroidogenic P450s. These sequences may differ among multiple P450 encoding genes and between genes in different species. *CYP11B2* gene expression is amplified by angiotensin II and



potassium ions, which increases intracellular calcium ions rather than the regulation of SF-1. Regulation and expression of 17 $\beta$ HSDs exhibit species-specific differences, due to distinct mechanisms controlling cell- and tissue- specific regulation (Payne and Hales, 2004).

### **2.5.5 Transport of synthesized steroid hormones**

Newly synthesised steroids are secreted directly into circulation where they are bound to specific proteins. Some hormone transport proteins in plasma are highly selective by having substrate specific characteristics. Sex hormone binding globulin (SHBG) transports estradiol, testosterone and DHT. Corticosteroid binding globulin (CBG) transports the major corticosteroid precursors, prog and 17OHprog. SHBG and CBG have high binding affinities with low binding capacities for their steroids. Albumin, the abundant plasma protein found in vertebrates, as well as other transport proteins, are comparatively non-selective and transport nearly all lipophilic molecules entering the bloodstream. Albumin is not strongly attracted to any one molecule in particular, therefore having low binding affinities but it does have a high binding capacity for steroids. (Baker, 2002).

All circulating steroids are mostly bound to transport proteins but a small group are unbound or free. Free steroids are available to act on target cells and are also serve as substrates for steroid metabolism in the peripheral tissues. SHBG and CBG contribute to the regulation and availability of steroid hormones for biological actions and their clearance (Baker, 2002).

### **2.6 CYP17A1: an exquisite regulator of steroidogenesis**

CYP17A1 catalyses two mixed-function oxidase reactions and therefore functions as a key branch point in steroidogenesis, determining which class of steroid hormones a given cell can produce (Miller and Auchus, 2011). The 17 $\alpha$ -hydroxylase activity is essential for the biosynthesis of C21-glucocorticoids, while both the 17 $\alpha$ -hydroxylase and 17,20-lyase activities are required for the production of C19-androgens. CYP17A1 is expressed in the zona fasciculata and -reticularis of the adrenal cortex. Zona glomerulosa lacks CYP17A1 and therefore, as a result, produces mineralocorticoids.

Several factors play a role in modulating CYP17A1 hydroxylase and lyase activity, which include levels of gene expression, the availability of different endogenous substrates and most importantly, the

presence of redox partners CPR and CYB5 (Gower and Cooke, 1983; Matteson *et al.*, 1986; Couch *et al.*, 1986).

### 2.6.1 The classic dual reactions

It was first believed that the dual reactions were carried out by two different enzymes (Nakajin *et al.*, 1981a); however, the authors demonstrated that a particular purified protein catalysed both 17 $\alpha$ -hydroxylation and C17-20 cleavage (Nakajin *et al.*, 1981b). This was confirmed by Zuber *et al.* (1986), by the subsequent cloning of CYP17A1 from bovine cDNA and its expression in COS-1 cells catalyzing both C21 and C21 17 $\alpha$ -hydroxylated steroids (Zuber *et al.*, 1986).

In adrenal steroidogenesis, the first CYP17A1 catalysed reaction is the 17 $\alpha$ -hydroxylation of the  $\Delta$ 5- and  $\Delta$ 4 C21 steroids, preg and prog, yielding their hydroxylated intermediates 17OHpreg and 17OHprog, respectively. Sequentially, the second reaction of CYP17A1 follows with the scission of the C17-20 bond to form the C19 steroids, DHEA and A4, respectively. The favoured substrate for 17,20-lyase activity is, however, species-specific. Each reaction involves one molecule of NADPH and one molecule of molecular oxygen (Payne and Hales, 2004). Figure 2-9 shows an overview of general CYP17A1 catalysed reactions.

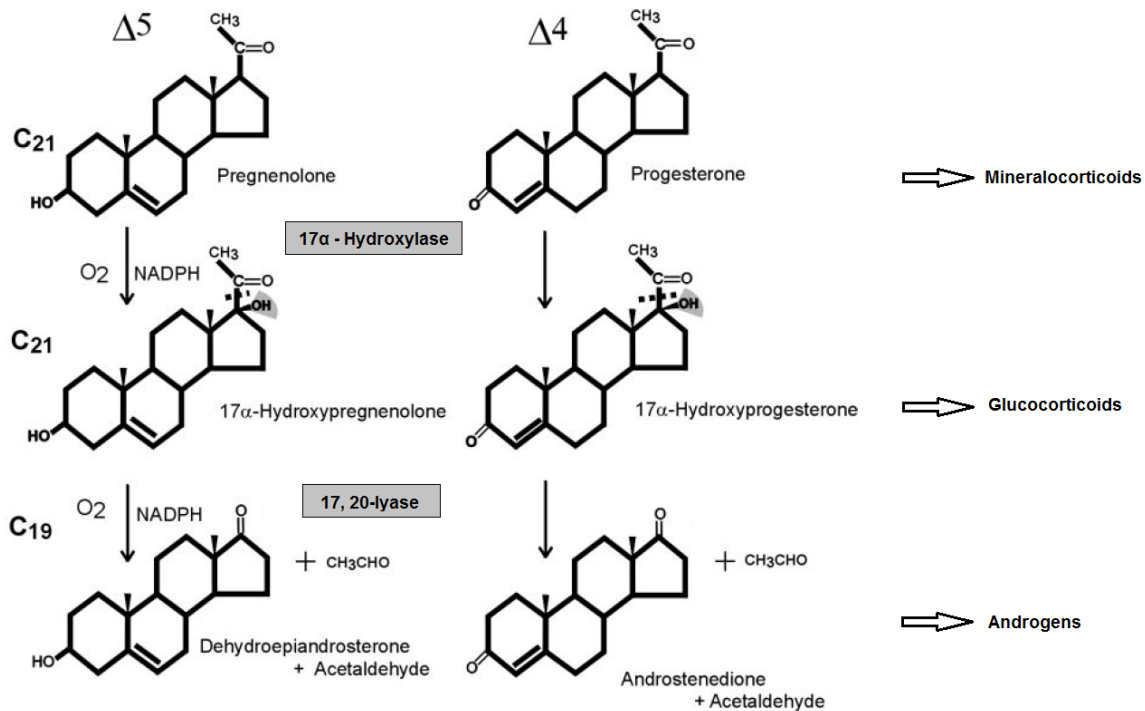


Figure 2-9: CYP17A1 catalysed reactions of the  $\Delta^5$ - and  $\Delta^4$  pathway. Preg and prog are two intermediates vital to synthesis of mineralocorticoids, glucocorticoids and androgens. CYP17A1 exhibits 17 $\alpha$ -hydroxylase activity in the conversion of C<sub>21</sub> steroids, preg and prog, to hydroxylated intermediates and 17,20-lyase activity involving the scission of the C<sub>17</sub>-C<sub>20</sub> bond of the hydroxy intermediates to produce DHEA and A<sub>4</sub>, respectively. Acetaldehyde is released as a by-product of the lyase reaction (Miller and Auchus, 2011).

The dual reactions catalysed by CYP17A1 are differentially regulated in a cell- and tissue-specific manner. Like all P450s, CYP17A1 requires CPR to shuttle electrons from NADPH to its heme centre (Auchus and Rainey, 2004). Using the microsomal methods derived from Nakajin *et al.* (1981a), Hall and colleagues demonstrated that by increasing the molar ratio of CPR to CYP17A1, a concomitant increase in the ratio of 17,20-lyase to 17 $\alpha$ -hydroxylase activity was observed in porcine testicular microsomes (Nakajin *et al.*, 1981a; Yanagibashi and Hall, 1986). They also illustrated, when compared to adrenal expression, that CPR was more plentiful in testes, where 17,20-lyase activity is required for the production of testosterone. These findings indicated that sufficient levels of CPR are essential for optimal 17,20-lyase activity. Throughout development, CPR is expressed in all three of the human adrenal zones, though the highest expression is in the zona reticularis and is dependent on the 17,20-lyase reaction for the production of C<sub>19</sub> androgen precursors (Suzuki *et al.* (2000).

In humans and chimpanzee, there is a prepubertal increase in adrenal secretion of C<sub>19</sub>-steroids (onset referred to as adrenarche). Principally, production of DHEA and its sulphate ester continually increases to levels exceeding cortisol secretion rates, reaching maximal levels at age 25-30 and slowly declining

thereafter (adrenopause) (Orentreich *et al.*, 1984). Adrenarche is independent of the gonads. Throughout life, most other mammals, including canine, equine, bovine, porcine, caprine and guinea pig species, all have relatively low DHEA-S serum concentrations and both old world and new world monkeys have rather high DHEA-S concentrations, which do not fluctuate as a function of age (Cutler *et al.*, 1978). Baboon and rhesus monkey DHEA-S concentrations are high at birth and decline shortly after (Sapolsky *et al.*, 1993). Although adrenarche has only been documented in humans and chimpanzees, fragmentary data suggests it may also occur in gorillas and orangutans (Cutler *et al.*, 1978). Adrenarche is thus characterized by the important feature of an increased conversion of 17OHpreg to DHEA, which is resultant of increased 17,20-lyase activity. At adrenarche, CPR expression increases in all zones of the adrenal. This in turn increases both activities of the CYP17A1 enzyme system, particularly the 17,20-lyase, enhancing the overall capacity of DHEA production (Auchus and Rainey, 2004). However, increased CYB5 expression in the zona reticularis during adrenarche is one of the major contributors to increased lyase activity.

Abnormal CYP17A1 function has been linked to a several diseases, including polycystic ovary syndrome (Qin and Rosenfield, 1998; Strauss, 2003), Cushing's syndrome (Ogo *et al.*, 1991), CAH (Maitre and Shirwalkar, 2003) and prostate cancer (Lunn *et al.*, 1999; Madigan, 2003). 17 $\alpha$ -Hydroxylase deficiency is a rare form of CAH. According to the P450 single gene expression system, the adrenals and gonads share the same CYP17A1 defect. The lack of adrenal 17 $\alpha$ -hydroxylase activity is characterized by weakened cortisol production and resultant hypersecretion of ACTH, which drives massive overproduction of the 17-deoxysteroids, deoxycorticosterone and corticosterone. Excess deoxycorticosterone suppresses the regulatory renin-angiotensin system, consequently, suppressing aldosterone synthesis causing hypertension and hypokalemia (Costa-Santos *et al.*, 2004). Defunct CYP17A1, exhibiting no hydroxylase activity is deficient of lyase activity as well, suppressing sex steroid formation. An absence of CYP17A1 lyase activity in humans, due to preferential congenital mutations, leads to the development of abnormal secondary sex characteristics, sexual infantilism in females and failure of virilization in males (Suzuki *et al.*, 1998). The 17,20-lyase activity is particularly sensitive to alterations in the interactions between CYP17A1 and its cofactors proteins CPR and CYB5 (Gupta *et al.*, 2001).

### 2.6.2 Species-specific CYP17A1 activities

The mechanism of action of these enzymes in the steroid metabolic pathway is complex and steroid metabolism in different species in the adrenal is continuously being investigated. Various species CYP17A1 show different selectivities towards substrates of steroid precursors and intermediates. A major difference between species CYP17A1 functions, is attributed to the lyase activity towards  $\Delta 5$ - and  $\Delta 4$  hydroxylated intermediates and the fact that in some species the secretion of C19-steroids is not restricted only to the gonads. Humans and higher primates can form DHEA and A4 in the adrenals. Lower vertebrates, such as rats, are incapable of synthesizing adrenal C19-steroids, as CYP17A1 is not expressed in the adrenals (Miller and Auchus, 2011).

Transfections in non-steroidogenic COS-1 cell lines, demonstrated that the initial hydroxylase reaction catalysed by human, bovine or rat CYP17A1 shows equal  $\Delta 5$ - and  $\Delta 4$  activities and that these species proteins can be differentiated on the basis of lyase activity (Fevold *et al.*, 1989). Human, bovine and porcine CYP17A1 favours 17OHpreg as the substrate for the lyase reaction (Miller, 1988), while the rat enzyme favours 17OHprog (Fevold *et al.*, 1989).

Human and baboon CYP17A1 cDNA share 96% amino acid sequence identity. Sequence analysis showed that there are 28 different amino acid residues between human and baboon CYP17A1, primarily in the F and G helices and the F-G loop (Swart *et al.*, 2002). Human and chimpanzee CYP17A1 differ by two amino acids, sharing more than 99% amino acid sequence identity (Arlt *et al.*, 2002). Baboon CYP17A1 has a significantly higher activity for prog hydroxylation relative to preg, whereas human and chimpanzee CYP17A1 favours preg hydroxylation (Arlt *et al.*, 2002). Baboon CYP17A1 has no 17,20-lyase activity for 17OHprog and although A4 forms part of the major androgens secreted by the human adrenal, human CYP17A1 17,20-lyase activity primarily converts 17OHpreg to DHEA via the  $\Delta 5$  pathway. Adrenal A4 is produced by the subsequent conversion of DHEA to A4 by 3 $\beta$ HSD2 (Swart *et al.*, 2002; Nakamura *et al.*, 2011). In species such as the rat and trout, testosterone synthesis is dependent on the  $\Delta 4$  pathway, while humans rely on the  $\Delta 5$  pathway (Brock and Waterman, 1999). Guinea pig CYP17A1 favours  $\Delta 4$  hydroxylase and -lyase activities and is incapable of converting 17OHpreg to DHEA (Tremblay *et al.*, 1994). Porcine and hamster CYP17A1 catalyse both  $\Delta 5$ - and  $\Delta 4$  hydroxylase and -lyase reactions (Zhang *et al.*, 1992; Cloutier *et al.*, 1995).

### 2.6.2.1 16 $\alpha$ -hydroxylase activity

In addition to 17 $\alpha$ -hydroxylase and 17,20-lyase activities, CYP17A1 also exhibits species-specific steroid hydroxylase activities. In some species, CYP17A1 exhibits 16 $\alpha$ -hydroxylase activity towards prog. In 1965, Sharma *et al.* (1965) first detected high levels of 16OHprog using microsomal studies involving testicular tissue incubated with prog (Sharma *et al.*, 1965). It was subsequently shown, that the 16OHprog produced from prog in human testicular microsomes, was not metabolized further and that it had an inhibitory effect on CYP17A1 lyase activity (Inano and Tamaoki, 1978). Further studies showed the conversion of prog to 16OHprog in the fetal adrenal (Yamasaki and Shimizu, 1973) and human ovaries (Sano *et al.*, 1981).

Both the human and chimpanzee enzymes convert striking amounts of prog to 16OHprog (Swart *et al.*, 1993; Arlt *et al.*, 2002). The C16 hydroxylation is catalysed in a common active site with the 17 $\alpha$ -hydroxylase and 17,20-lyase reactions. This enzyme does not hydroxylate preg at the C16 position (Swart *et al.*, 1993).

Human CYP17A1 has been shown to produce a 16OHprog to 17OHprog ratio of 1:4 when expressed in COS-1 cells (Swart *et al.*, 1993) and 1:2 when expressed in yeast (Storbeck *et al.*, 2008a). Even though the physiological importances of mineralocorticoids, glucocorticoids and sex steroids have been well established, the precise function of 16OHprog is yet to be determined.

16 $\alpha$ -Hydroxylase activity has been reported in other primate and mammalian species, although levels of 16OHprog are substantially lower than in human and chimpanzee (Swart *et al.*, 1993; Arlt *et al.*, 2002). Arlt *et al.* (2002) demonstrated the ratio, of 16 $\alpha$ -hydroxylase to 17 $\alpha$ -hydroxylase activities of human and chimpanzee CYP17A1, to be 8-fold higher than that of baboon CYP17A1 and 2-fold higher than rhesus monkey CYP17A1 (Arlt *et al.*, 2002). Storbeck *et al.* (2008a) developed a sensitive UPLC-coupled atmospheric pressure chemical ionization mass spectrophotometry assay, which enabled the detection of 16OHprog, produced by the angora goat CYP17A1 expressed in COS-1 cells (Storbeck *et al.*, 2008a). The authors concluded that although some species did produce small amounts of 16OHprog, the levels were below the previous limits of detection.

A study involving site-directed mutagenesis and prog conversion assays in COS-1 cells, identified a single amino acid residue responsible for conferring 16 $\alpha$ -hydroxylase activity in human CYP17A1, namely Ala at position 105 (Ala105) (Swart *et al.*, 2010). They demonstrated that introducing the

mutation Ala105Leu into the human enzyme significantly increased the ratio of 17OHprog to 16OHprog when compared to the wt enzyme. Conversely, introducing the Leu105Ala mutation into the baboon, caprine and porcine enzymes resulted in a decreased ratio of 17OHprog to 16OHprog when compared to the respective wild-types (Swart *et al.*, 2010).

#### 2.6.2.2 16-ene synthase activity

CYP17A1 possesses the ability to catalyse the formation of a third class of active steroids, referred to as 16-unsaturated steroids or 16-androstenes, via its 16-ene synthase activity (Soucy *et al.*, 2003). Synthesis of 16-ene steroids are highly species-specific and are based on the nature and functions of these steroids. These steroids are sex pheromones and are produced predominantly by the gonads in humans and porcine (Weusten *et al.*, 1989). Two types of 16-androstenes, 5 $\alpha$ -androst-16-en-3-one (androstenone) and 5 $\alpha$ -androst-16-en-3 $\alpha$ (or  $\beta$ )-ol (androstenols), have aroused considerable interest as they elicit both primer and releaser pheromonal effects in porcine, predominantly androstenone, which is stored in adipose tissue of mature boars (Pattersen, 1968). 16-Androstenes are present in neonatal porcine testis and the quantities increase progressively with age, particularly after 18 weeks (Booth, 1975).

Production of 16-androstene steroids is catalysed by CYP17A1s 16-ene synthase activity, which requires the presence of CYB5: together CYP17A1 and CYB5 are referred to as the andien- $\beta$  synthase system (Katkov and Gower, 1970). In species producing androstene steroids, the CYP17A1-CYB5 enzyme system is capable of cleaving the C17,20 carbon bond of steroid substrates without prior 17 $\alpha$ -hydroxylation. Overproduction of 16-androstene steroids by the testis can be attributed to the high levels of CYB5 found in testicular tissue (Robic *et al.*, 2008).

Lin *et al.* (2005a) developed a sensitive and flexible single-stranded conformational polymorphism (SSCP) technique, which is capable of detecting a single nucleotide polymorphism (SNP) (Lin *et al.*, 2005a). They used this method to assess the result of a tyrosine to adenosine nucleotide substitution at 1317 bp of porcine CYP17A1 nucleotide gene sequence. The mutation caused a Leu439 to His439 amino acid substitution and no changes were observed in CYP17A1 enzyme activity in the biosynthesis of androstenone or sex steroids. Other polymorphisms (formerly suggested to be imperative for the functional interaction of CYP17A1 with CYB5 in human) were not observed (Lin *et al.*, 2005a). The synthesis of androstenone in porcine testis was not affected by any polymorphisms in the coding region



of the porcine CYP17A1 gene. The authors also identified a guanine/tyrosine SNP in porcine CYB5 mRNA at base 8, upstream to the ATG in the 5'-untranslated region (Lin *et al.*, 2005b). Of the total 229 testis samples tested, 84.8% were homozygous for the guanine variant, 12.4% were heterozygous, and 2.8% were homozygous for the tyrosine variant. The latter demonstrated significantly lower CYB5 activity than variant guanine, as well as a subsequent reduction in androstenone production.

Studies by Kwan *et al.* (1985) showed C21 steroids are substrates for 16-ene activity and the first metabolites formed are androstadienol (andien- $\beta$ ), synthesized from prog and androstadienone, synthesized from prog (Kwan *et al.*, 1985). The 16-ene synthesis pathway in immature porcine testicular microsomes is shown in Figure 2-10 together with the key steroid enzymes, CYP17A1, CYB5, 17 $\beta$ -, 3 $\beta$ HSD and 5 $\alpha$ -reductase, which are involved in 16-ene biosynthesis.

CYP17A1 appears to be capable of cleaving the C17,20 bond of prog directly in a species-specific manner and requires the assistance of CYB5. Prog can be converted into androstadienone but not via 17OHprog or 16-dehydroprog as intermediates (Kwan *et al.*, 1984). Neither 17OHpreg (Katkov and Gower, 1970) nor 17OHprog (Ahmad and Gower, 1968) can be converted to 16-androstenes directly. Kwan *et al.* (1984) found DHEA and A4 present from the typical 17,20 cleavage of 17OHpreg and 17OHprog, respectively (Kwan *et al.*, 1984). The concentration of A4 was 31 times higher than the control incubation. The high proportion of 17OHprog being metabolized by 17,20-lyase, may explain why it is not available for the conversion to 16-dehydroprog and androstadienone. The authors base the validity of such an explanation on the assumption that CYP17A1 is active and capable of catalyzing the direct conversion of prog to the 16-androstene precursors in neonatal porcine testis (Kwan *et al.*, 1984). No androstenes were formed when 16OHprog was used as a putative precursor (Kwan *et al.*, 1985). A similar conclusion was reported by Matsui and Fukushima (1970) who used 16OHprog and boar testis preparations (Matsui and Fukushima, 1970).



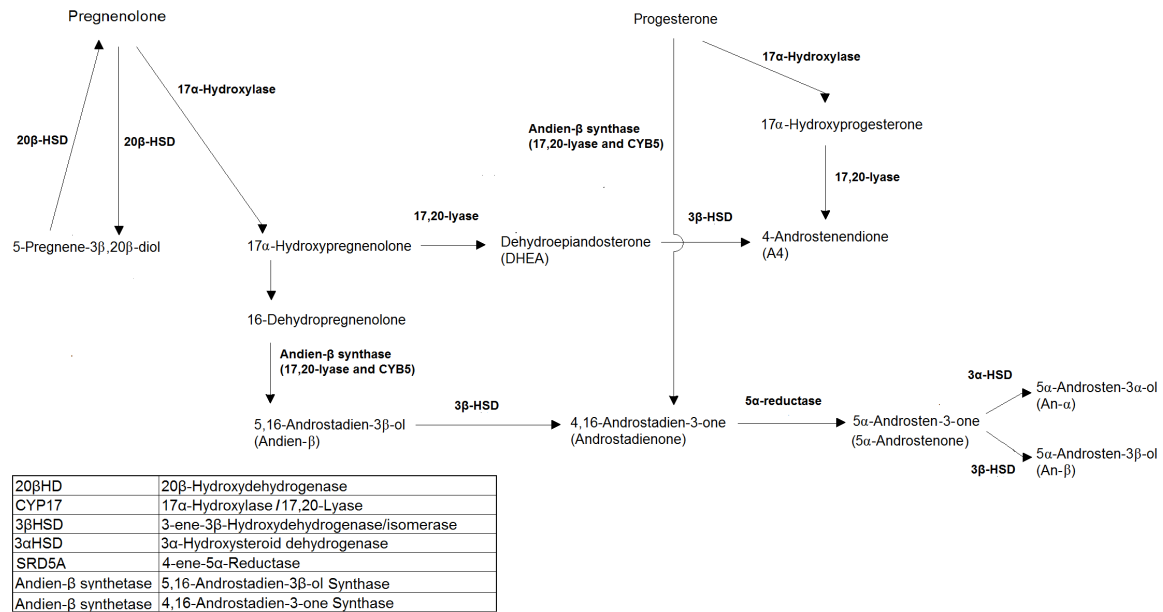


Figure 2-10: CYP17A1 16-ene synthase activity in neonatal porcine testicular microsomes. CYB5 and 3βHSD are essential for 16-ene steroid synthesis. Species-specific 17,20-lyase activity, in the presence of high levels of CYB5, is responsible for conferring andien-β synthase activity. Preg and prog are the major substrates for andien-β synthase action; they produce the first 16-ene precursor steroids, andien-β and androstadienone, respectively. It appears that a single direct side-chain cleavage reaction of prog is responsible for androstadienone formation, while 17OHpreg and 16-dehydropreg are intermediates in andien-β synthesis. 5α-reductase and 3β-HSD are involved in formation of final 16-androstene products (adapted from Kwan *et al.*, 1984).

Mason *et al.* (1979) first proposed that prog is converted into andien-β via a pathway first involving 17α-hydroxylation of prog, then a dehydration reaction forming 16-dehydropreg with andien-β synthase subsequently catalyzing the side-chain cleavage (Mason *et al.*, 1979). Kwan *et al.* (1984) confirmed this and added that the final androstenol products androsten-3α-ol (An-α) and androsten-3β-ol (An-β) are formed by andien-β metabolism; see Figure 2-11 (Kwan *et al.*, 1984).

Loke and Gower (1972) suggested that 5-pregnen-3β,20β-diol was also an intermediate in andien-β synthesis from preg in boar testicular microsomes (Loke and Gower, 1972). However, when 5-pregnen-3β,20β-diol was given as a substrate, 17OHpreg and 16-dehydropreg were found as well as andien-β. They suggested/concluded that the diol was converted back to preg by 20β-hydroxysteroid dehydrogenase (20βHSD) and then metabolized to andien-β (Loke and Gower, 1972).

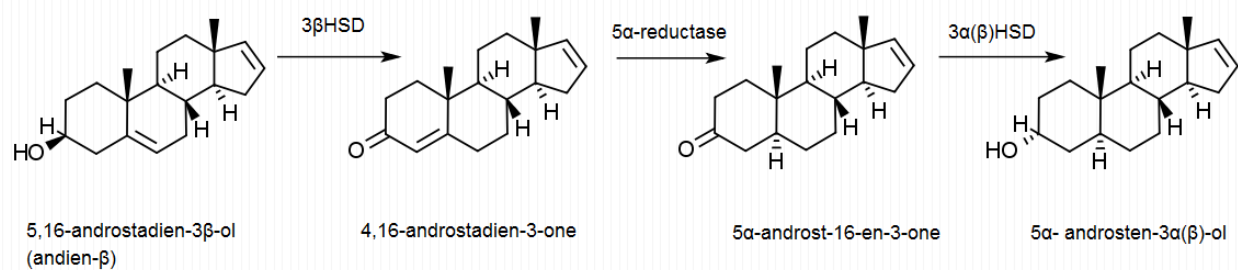


Figure 2-11: Andien-β metabolism and the formation of 16-androstene steroids. The 16-ene synthase complex, porcine CYP17A1 and CYB5, transforms prog into andien-β and prog into androstadienone.

Andien-β is transformed via 3βHSD activity, yielding the steroid intermediate androstadienone, which is then reduced by 5α-reductase to yield androstenone (Kwan *et al.*, 1984). Androstenone is finally reduced to α- or β-androstenol, by 3α- or 3βHSD, respectively (Robic *et al.*, 2008). 3βHSD is active in both the liver and testis but the expression of the hepatic form is negatively correlated with androstenone levels in adipose tissue. In comparison with other species, variations detected in the promoter region of porcine *HSD3B* gene do not show any relationship with androstenone formation but are associated more with the type of breed (Cue *et al.*, 2007). Cue *et al.* (2007) speculated that transcription factors, responsible for the liver-specific regulation of the *HSD3B* expression, may be crucial for regulating the manner in which androstenone is deposited in porcine adipose tissue (Cue *et al.*, 2007).

It is clear that CYP17A1 together with CYB5, as well as 3βHSD are crucial to the production of 16-ene steroids.

## 2.7 Cytochrome b5: a precise modulator of steroid enzyme activity

In mammals, CYB5 exists as a soluble cytosolic protein (also found in erythrocytes) or as a membrane-bound protein, the latter acting as either a modifier or an essential component of P450 or non-P450 enzyme catalysed reactions in steroid biosynthesis (Schenkman and Janssom, 2003). Membrane-bound CYB5 are classified as two types, according to primary structure and sub-cellular localization. The first type is a 134 amino acid protein, localized to the ER and the second type is the 146 amino acid protein, localized to the OMM (Ito, 1980; Lederer *et al.*, 1983). The expression of both CYB5 isoforms have been detected in a variety of tissues, including the adrenals, gonads, kidney, liver, fat and brain (Giordano and Steggles, 1991; Giordano and Steggles, 1993).

Two genes express human CYB5. The first gene on chromosome 18q23 has 6 exons that undergo alternative splicing: exons 1-4 encode a 98 amino acid soluble form and exons 1-3 and 5-6 encode the widely expressed 134 amino acid CYB5 form bound to the ER (Giordano *et al.*, 1993). The second gene on chromosome 16q22.1 incorporates 5 exons, which encode the 146 amino acid OMM-bound form (Kuroda *et al.*, 1998). The 134 amino acid ER-bound protein is the only form known to have significant effects on adrenal steroidogenesis. It is the most dominant CYB5 isoform present in the adrenal and is localized to the zona reticularis, the site of adrenal androgen production, where its expression coincides with the onset of adrenarche (Miller and Auchus, 2011).

The 134 amino acid ER-bound form of CYB5 is a 16 kDa electron transfer protein consisting of three structural domains: the N-terminal and heme-containing soluble domain, a C-terminal membrane anchor and a linker region, which connects the two domains (Renthal, 2010). The amino acid sequence of the truncated catalytic heme domain is highly conserved throughout all species, although the full-length protein shows over 80% amino acid sequence identity in mammals (Ozolc, 1989). The heme domain (~90 amino acids) is responsible for electron transfer and possesses several negatively charged residues, which are involved in the binding of CYB5 to its redox partners (Im and Waskell, 2011). Of the 28 amino acids comprising the N-terminal tail, 23 are hydrophobic. It is unclear whether the tail domain interacts with P450s or if they are only necessary for the appropriate positioning of the heme group. The last 10 residues of the C-terminal are essential for targeting the ER and for membrane insertion (Mitoma and Ito, 1992). A study showed that the insertion of additional residues in this region caused the protein to be transported to the plasma membrane of COS-1 cells (Honsho *et al.*, 1998). The linker domain (~15 amino acids) is highly flexible and is crucial for heme positioning in the optimal orientation to stimulate the P450. Proteolytic cleavage before or after the linker domain disassociates the soluble catalytic domain from the membrane anchor. Soluble CYB5 cannot modify the activities of the microsomal steroidogenic enzymes, with only the membrane bound forms being able to perform this function (Schenkman and Janssom, 2003).

### **2.7.1 An allosteric regulator of 3 $\beta$ HSD**

During the biosynthesis of steroid hormones, 3 $\beta$ HSD competes with CYP17A1 for  $\Delta^5$  steroid intermediates. The ratio of the enzyme activities and substrate specificities are critical in determining the level of production of steroid intermediates through the pathway (Conley and Bird, 1997). This competition is significantly altered by CYB5, which selectively stimulates CYP17A1 lyase activity and

consequently increases C19 steroid production substantially (Katagiri *et al.*, 1982). Goosen *et al.* (2011) investigated the influence of CYB5 on 3 $\beta$ HSD activity and showed in COS-1 cells, co-transfected with 3 $\beta$ HSD and CYB5, that the latter significantly increased both caprine and ovine 3 $\beta$ HSD activity towards preg, 17OHpreg and DHEA in a substrate and species-specific manner (Swart *et al.*, 1995; Goosen *et al.*, 2011). Although the  $K_m$  values remained unchanged, CYB5 increased the apparent  $V_{max}$  values of the enzyme for all substrates. In a follow up study, Goosen *et al.* (2012) showed that CYB5 augments 3 $\beta$ HSD activity via an allosteric mechanism by increasing the affinity of the enzyme for cofactor NAD<sup>+</sup> (Goosen *et al.*, 2012).

### 2.7.2 An amplifier of 17,20-lyase activity

CYP17A1 lies at the junction of androgen and corticoid biosynthesis: hydroxylation being common to both pathways and the carbon-carbon bond cleavage only necessary for androgen formation (Nakajin *et al.*, 1981b). Under normal physiological conditions, there must be precise control mechanisms preventing the hydroxysteroids from undergoing increased carbon-carbon bond cleavage as observed in excessive androgen production in the adrenal gland contributing to virilization (Miller and Auchus, 2011). A remarkable positive correlation is shown between DHEA production and the significant co-localization of CYB5 and CYP17A1 in the ER of the gonads and the adrenal zona reticularis and implicated the involvement of CYB5 as a potential modulator of the cleavage activity of CYP17A1 (Lu *et al.*, 1974).

There is a significant amount of research dedicated to demonstrating what a profound influence CYB5 has on CYP17A1 activity. Many sources data show (especially for species with minimal to no lyase activity), that optimal molar ratios of CYB5 to CYP17A1 increases the rate of the 17,20-lyase reaction, to the extent that that it approaches the rate of the hydroxylase reaction (Katagiri *et al.*, 1982; Kominami *et al.*, 1992; Lee-Robichaud *et al.*, 1995b; Auchus *et al.*, 1998).

In 1982, Hall and colleagues were the first to show the dramatically enhanced 17,20-lyase activity of CYP17A1 in the presence of purified CYB5 protein (Onoda and Hall, 1982). Katagiri *et al.* (1982) demonstrated the selective stimulation of CYP17A1 lyase activity in the presence of CYB5, which was stimulated up to 10-fold, with insignificant stimulation of the hydroxylase activity (Katagiri *et al.*, 1982). The authors concluded that the degree of side-chain cleavage was dependent on the concentration of CYB5.

Auchus *et al.* (1998) established that CYB5 increases 17,20-lyase activity by acting as an allosteric effector on the CYP17A1-CPR complex and facilitating optimal electron transfer from CPR to CYP17A1 (Auchus *et al.*, 1998).

Lee-Robichaud *et al.* (1995b) showed that human CYP17A1 differs from other mammalian enzymes with the carbon-carbon bond cleavage activity exhibiting an absolute requirement for CYB5 (Lee-Robichaud *et al.*, 1995b). They concluded that the low levels of CYB5 found in the adrenal cortex should be an important factor in limiting the formation of androgens by this tissue. In the same study, the authors made use of NADPH-CPR as the electron donor and showed that homogenous porcine CYP17A1 catalysed the conversion of preg and prog to their 17 $\alpha$ -hydroxylated counterparts, which were subsequently cleaved into androgens through the enzyme's lyase activity. The study showed that CYB5 stimulated both these activities; however its most noticeable effect was in the formation of 16-ene steroids, which require CYB5 for their production (Lee-Robichaud *et al.*, 1995b).

### 2.7.3 CYB5 structure-function model

CYB5 belongs to a group of widespread integral proteins with a monotopic membrane topology with the N-terminal of the protein presenting itself on the cytosolic side of the membrane and the C-terminal incorporated within the membrane layers (Khaderbhai *et al.*, 2003).

Lee-Robichaud *et al.* (1997) determined the significance of the membrane-anchoring domain in altering CYP17A1 lyase activity using modified forms of rat CYB5 protein: i) native, wt (core-tail, residues 1-134); ii) globular, soluble domain (core, residues 1-99); iii) wt attached to an alkaline phosphatase signal sequence at the N-terminal (signal-core-tail, residues 21-134) and iv) the globular core attached to the alkaline phosphates signal sequence (signal-core, residues 21-99) (Lee-Robichaud *et al.*, 1997). The authors proposed a topology-based membrane model of CYB5 with CYP17A1 and is shown in Figure 2-12. Each variant of CYB5 was constructed in such a manner that when anchored in the membrane, the variant would impose a different orientation of the globular domain for its interaction with CYP17A1. Native CYB5 is favourably positioned for optimal interaction with CYP17A1 (Figure 2-12a). Signal-core-tail variants were able to adopt three topological states (Figure 2-12c and d), though only one could provide dynamic interactions with CYP17A1 (Figure 2-12c, *right*). Core and signal-core variants (Figure 2-12b and c, *left*) were unsuitably orientated with respect to CYP17A1.

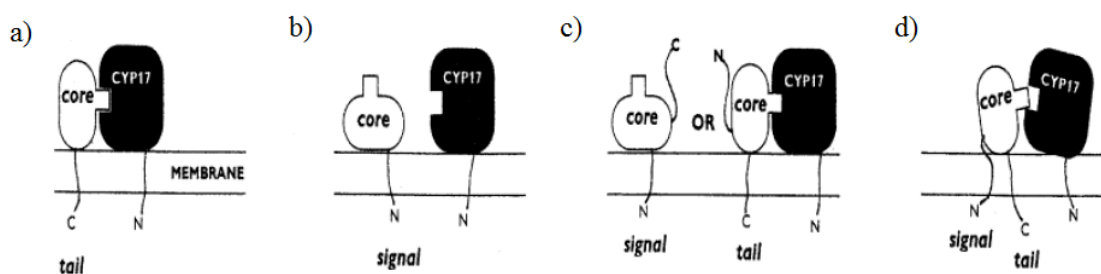


Figure 2-12: Model depicting the possible topologies of membrane bound variants of CYB5 and CYP17A1. (a) Native (wt) CYB5 anchored to the membrane via a C-terminal domain (core-tail) shows optimal interaction with CYP17A1. (b) No positive interaction is achieved between CYP17A1 and the globular, core. Signal-core variant does not possess the C-terminal membrane anchor and is N-terminally attached to membrane via alkaline phosphatase signal sequence. Signal-core-tail variants can present in three topological states: the first state (c, left) allows for no interaction; the second state (c, right) allows for a functional interaction and the last state (d) allows for a weak interaction. The authors excluded CPR from this model for clarification purposes (reproduced from Lee-Robichaud *et al.*, 1997).

Native rat CYB5 stimulates maximum lyase potential (Katagiri *et al.*, 1982; Auchus *et al.*, 1998) and it was postulated to be as a result of optimal interaction and/or binding between CYB5 and CYP17A1 (Lee-Robichaud *et al.*, 1997). The C-terminal orientates the globular portion in the favoured position with the associated binding site in CYP17A1, therefore ensuring optimal activity. The signal-core-tail CYB5 variant exhibited a 2-fold lower affinity for CYP17A1 and ~55% of the lyase stimulatory activity by native CYB5. Signal-core and core variants of CYB5 were incapable in promoting lyase activity due to an inadequate orientation with respect to CYP17A1. The authors concluded that the decreased catalytic competencies, imposed by CYB5 variants with modified tail domains, were attributed to their modified interactions with CYP17A1. The incapacity of the soluble heme core to stimulate the lyase reaction is corroborated by other reconstituted CYP-CPR-CYB5 studies (Lamb *et al.*, 2001; Clarke *et al.*, 2004). This suggests that the membrane anchor domain provides the appropriate membrane-integrated topology of CYB5 for maximum interaction in a quaternary complex with CYP17A1, as well as contributing to the stability of the protein-protein interface through amino acid residue charge-based pairings and non-specific hydrophobic interactions (Mulrooney *et al.*, 2004).

## 2.8 Mechanism of CYP17A1 activity

### 2.8.1 Redox partners, electron transfer and heme reduction

The first oxidation reaction catalysed by CYP17A1 requires the transfer of two electrons, via a redox partner, for the fundamental reduction of the proteins oxyheme group. CPR is the typical redox partner

for P450s and is abundant in mammalian cells, contributing to the transfer of both electrons from NADPH to CYP17A1.

Auchus *et al.* (1998) investigated the amplification of CYP17A1 activity in reconstituted assays by means of variable molar ratios of CYB5 to CYP17A1 while CPR concentration were maintained (Auchus *et al.*, 1998). The authors observed maximum stimulation of the lyase reaction when ratios between CYB5 and CYP17A1 varied from 10:1 to 30:1. CYB5 in excess, rapidly and considerably decreases augmentation of the lyase reaction, which suggests that at these high levels of CYB5, electron scavenging from CPR occurs, reducing the potential of the initial electron to reduce CYP17A1. They investigated the function of CYB5, using genetically modified CYB5 protein that was devoid of heme (apo-CYB5). The expressed recombinant apo-protein, exhibited a similar stimulatory effect on CYP17A1 lyase activity, when compared to the holo-protein. The lyase activity was not repressed when the ratio of apo-CYB5 to CYP17A1 concentrations exceeded the optimal ratio of 30:1 for expressed holo-CYB5 (Auchus *et al.*, 1998). Estabrook (1999) proposed that during the reduction of CYP17A1 in the second catalytic reaction, the second electron can be derived from CYB5, where CYB5 functions as a substitute redox partner to the conventional CPR (Estabrook, 1999). The quicker rate at which the second electron is transferred from CYB5 to CYP17A1 is thought to reduce the possibility of spontaneous decay of iron-oxygen complex during the P450 oxidoreduction cycle (Estabrook, 1999).

However, in accordance with the findings from Auchus *et al.* (1998), additional studies led to the understanding that CYB5 does not play a role in electron donation or transfer but rather possesses remarkable allosteric capabilities in modulating CYP17A1 activity (Auchus *et al.*, 1998; Lee-Robichaud *et al.*, 1998; Schenkman and Jansson 2003). It was proposed that CYB5 serves as an allosteric modulator, which induces a conformational change within the enzyme that is sufficient in promoting optimal interaction between CPR and CYP17A1, thus enhancing the flow of electrons within the system via CPR-flavin domains, as well as to promote the disassembly of the CYP-substrate complex in the catalytic cycle (Schenkman and Jansson, 2003).

### **2.8.2 Redox partners and complex formation are essential for CYP17A1 activity**

CPR, a diflavin reductase, is a ~82 kDa membrane-bound protein, which constitutes 680 amino acids and utilizes both flavins, FMN and FAD, as cofactors. They bind to different domains in CPR and



serve as intermediates in the transfer of electrons from NADPH (Narayanasami *et al.*, 1995; Shephard *et al.*, 1989). Human and other species CPR, share highly conserved binding domains for FMN, FAD and NADPH. FAD and NADPH domains are similar to the FAD and NADPH binding domains of *fdx*. The cofactor binding domains are connected by a flexible, hinge region consisting of ~10-12 amino acid residues (Pandey and Flück, 2013). Comparable to microsomal P450s, CPR is membrane-bound to the ER by a N-terminal hydrophobic anchor, which consists of ~55 amino acids. Earlier studies showed that recombinant CPR devoid of the membrane anchor, was unable to support P450-mediated reactions as the removal of this N-terminal sequence led to a loss of interaction with P450s (Lu *et al.*, 1969). This emphasized the importance of the membrane anchor for the interaction with P450s.

Two mechanisms are suggested in playing a role in the association between redox partners and CYP17A1, namely electrostatic interactions between the anionic residues of CPR pairing up with cationic amino acid residues on the surface of the CYP17A1 molecule, as well as through non-specific interactions of the hydrophobic membrane regions of both proteins (Kurian *et al.*, 2004).

CYP17A1 complexes with CPR during the first hydroxylation reaction. Two electrons are received from NADH/NADPH and CPR facilitates the transfer by accommodating these two electrons and transferring them, separately and sequentially via FAD and FMN, to the P450. The CPR-P450 complex assumes a molecular alignment with respect to the substrate, which allows for oxidative attack by CYP17A1, which hydroxylates the substrate at C17 position (Akhtar *et al.*, 2005). CYB5 may either facilitate conventional CPR redox reactions (in CYP17A1 catalysed reactions) or as a donor of second electron to P450s (drug metabolizing P450s) (Shimada *et al.*, 2005). During the second catalytic round, species- and tissue-dependent, CYB5 may interact with the CYP17A1/CPR complex and induce a conformational change at the active site in CYP17A1s, which expedites an oxidative attack at C20 of the hydroxylated substrate intermediate, resulting in the dissociation of the bond between C17 and C20 (Lee-Robichaud *et al.*, 1998). No apparent or remarkable conformational changes were observed by CYB5 physically interacting with CYP17A1s active site, it was subsequently proposed that there are rather understated structural modifications that occur in the interior active sites of CYP17A1 (Akhtar *et al.*, 2005). Lee-Robichaud *et al.* (1998) proposed that the substrate intermediate, whilst still maintaining its affinity for the enzyme, was reorientated to a position, which favoured C17-C20 cleavage (Lee-Robichaud *et al.*, 1998). Mutations in CPR cause a complex set of disorders that often resemble defects in steroid metabolizing enzymes CYP17A1, CYP21A2 and aromatase (Flück *et al.*, 2007). Figure 2-13 shows allosteric modulation by CYB5 promoting CYP17A1 cleavage activity.



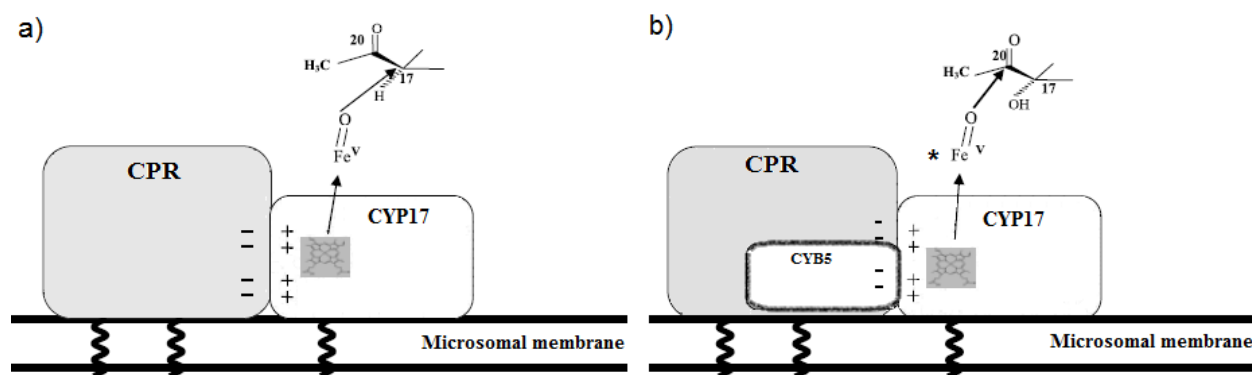


Figure 2-13: Model proposing CYB5-mediated changes in CYP17 (CYP17A1) 17,20-lyase activity. Two electrons are acquired from NADP and transferred by CPR and/or CYB5, which interact with CYP17A1 via electrostatic interactions between oppositely charged amino acid residues. Non-specific interactions between the membrane-anchoring regions and membrane facilitates the formation of the complex. (a) The CPR-CYP17A1 complex generates a molecular alignment that is sufficient for the oxidative attack of CYP17A1 to hydroxylate a steroid substrate at the C17 position and not at the C20. (b) CYB5 may interact with this complex during the second round of catalytic cycle and can induce a conformational change in CYP17A1 at the active site. The change will induce an oxidative attack by the oxyheme intermediate (\*) of CYP17A1 and redirect it towards the C20 of the hydroxylated substrate intermediate and is observed by the scission of the C17-C20 bond. Fe-O-O is suggested to be the attacking oxyheme intermediate (reproduced from Akhtar *et al.*, 2005).

In the CYP17A1-redox partner complex, C17 is in closer proximity with the activated oxyheme intermediate and hydroxylation is generally favoured at this carbon position; however it has been reported that this attack may also take place at the C16 position (Swart *et al.*, 1993). The CYP17A1-CPR-CYB5 complex alters CYP17A1 orientation by bringing the oxyheme intermediate into alignment with the C20 position of hydroxylated substrate intermediate, either through repositioning of the heme group or substrate intermediate. A consequent nucleophilic attack (cleavage) is directed towards the carbonyl group of the substrate to form a shorter steroid product and acetic acid (Lee-Robichaud *et al.*, 1998). In species with 16-androstene steroid production, the oxyhemeprotein-CYB5 complex causes a unique realignment of certain steroid substrates facilitating 16-ene synthase activity of CYP17A1 in bypassing prog hydroxylation and cleaving prog directly to yield androstadienol or in the formation of andien- $\beta$  from 16-dehydropreg cleavage (Soucy *et al.*, 2003). These reaction takes place at a rate 10-fold faster when in the presence of high amounts of CYB5 and occurs in Leydig cells.

## 2.9 Summary

The biochemical pathway that comprises all active adrenal and gonadal steroid hormone biosynthesis is exquisitely controlled. Steroidogenic enzymes are responsible for catalyzing the formation of each steroid intermediate and product and the extent of their activities are facilitated by electron transfer

and/or redox partner proteins. These enzymes are tightly regulated under HPA-axis control, as well as on levels of gene transcription, post-translational modifications and cell-, tissue- and species-specific expression patterns.

Cholesterol and its immediate C21 metabolite, preg, are the parent steroids from which all steroid intermediates are derived. P450s and HSDs play pivotal roles in steroid hormone biosynthesis. They have unique and specialized functions based solely on the substrates that they can catalyse. Preg initiates the  $\Delta^5$  steroid pathway in androgen and corticoid formation. Dehydrogenation/isomerisation of preg yields prog, which initiates the  $\Delta^4$  pathway in androgen and corticoid formation.  $\Delta^5$ - and  $\Delta^4$  androgen formation vary greatly in the adrenals among different species, particularly with A4 formation.

The enzyme CYP17A1 plays an essential role in both adrenal and gonadal steroidogenesis in mammals. CYP17A1 functions at intersections in competition with  $3\beta$ HSD for substrates. CYP17A1 exhibits dual activity of hydroxylation of C17 and side-chain cleavage of the carbon-carbon bond between C17,20 of C21 steroids, the latter reaction being strongly amplified by CYB5 in a species-specific manner.  $3\beta$ HSD and  $17\beta$ HSD exist as multiple isoforms and are crucial components in the biosynthesis of all steroids.  $3\beta$ HSD catalyses the transition between  $\Delta^5$ - and  $\Delta^4$  C21 steroids and together with the  $17\beta$ HSDs catalyze vital steps in C19 steroid formation. Recent publications have placed new emphasis on the steroid synthesis pathway through the allosteric interaction between  $3\beta$ HSD isoforms and CYB5 which augments the activity of  $3\beta$ HSD thus creating a highly competitive environment with CYP17A1 for substrates (Goosen *et al.*, 2011; 2012).

In addition to the conventional reactions of CYP17A1, CYP17A1 has species-specific  $16\alpha$ -hydroxylase and 16-ene-synthase activity. A recent publication has attributed  $16\alpha$ -hydroxylase to amino acid residue Ala105 (Swart *et al.*, 2010). CYP17A1, together with high levels of CYB5, also has the ability to cleave the C17,20 side chain of C21 steroids without prior  $17\alpha$ -hydroxylation and form pheromonal 16-androstenes. This initial lyase reaction is greatly species- and tissue-specific and purely dependent on CYB5.  $3\beta$ HSD is equally essential in this pathway and is responsible for final product formation. It is clear that CYP17A1 together with CYB5, as well as  $3\beta$ HSD are crucial to the regulation and formation of major steroids.

In the following chapter, sequence comparisons of the cDNA encoding two wt porcine CYP17A1's and the consequences of point mutations, assessed in terms of the catalytic activity towards prog, will be

presented. The influence of porcine CYB5, cloned from liver mRNA, as well as that of CYB5 from various other species on wt porcine CYP17A1 activity, will also be presented in subsequent chapters.

## CHAPTER 3

### THE INFLUENCE OF VAL40LEU, HIS407LEU AND LEU105ALA SUBSTITUTIONS ON 17 $\alpha$ -HYDROXYLASE/17,20-LYASE AND 16 $\alpha$ -HYDROXYLASE ACTIVITIES OF PORCINE CYP17A1

#### 3.1. Introduction

CYP17A1 cDNA, 1527 bp, encodes a protein of 509 amino acids and has a predicted molecular weight of 56 kDa (Chung *et al.*, 1987). This enzyme catalyses both the 17 $\alpha$ -hydroxylase and 17,20-lyase reactions, essential to steroidogenesis in the adrenal and gonads (Miller and Auchus, 2011). CYP17A1 also has the ability to catalyse the 16 $\alpha$ -hydroxylation of prog with Ala105 being the key residue in conferring this activity in humans (Swart *et al.*, 2010). CYP17A1 from various species exhibit markedly different activities because of structural variations, differences in redox partner binding and differences in the contribution of CYB5 to the lyase activity. The reactions are catalysed in a common active site (Miller and Auchus, 2011). Mutations in the gene encoding this protein result in the loss of a functional protein eliminating either the 17,20-lyase activity (Biason-Lauber *et al.*, 1997), 17 $\alpha$ -hydroxylase (Fardella *et al.*, 1994) or both activities (Suzuki *et al.*, 1998). Mutations with such consequences usually fall within conserved domains of the proteins three-dimensional (3D) structure (Chung *et al.*, 1987). Research has identified key amino acid residues within the CYP17A1 sequence, which are solely responsible for- or that have considerable influence on enzyme activity and essentially steroid product formation (Swart *et al.*, 2010; Gupta *et al.*, 2001).

The obstacles in structural studies of membrane-bound proteins are generally attributed to their high level of hydrophobicity, which makes them susceptible to misfolding and aggregation and their insolubility does not allow for obtaining adequate quality crystals using X-ray crystallography. Furthermore, their large size and slow reorientation does not make them suitable for structure determination using solid-state NMR spectroscopy (Lacapère *et al.*, 2007). However, since the first published structure in 1965, the number of structures determined for membrane proteins, have grown exponentially (Deisenhofer *et al.*, 1985; White, 2004). Additional methods have been developed in ascertaining 3D structures, one widely used technique is homology, comparative or molecular modelling (Kirton *et al.*, 2002). It has been observed that proteins with similar amino acid sequences

have a tendency to adopt similar 3D structures (Chothia and Lesk, 1986). Therefore, it is possible to predict the 3D structure (though inherently less accurate than those derived experimentally) of a protein based exclusively on knowledge of its amino acid sequence and the 3D structures of proteins with similar sequences.

Much has been learnt in terms of structure/function through enzyme deficiencies brought about by specific mutations occurring in patients as well as site-directed mutagenesis. The structure of human CYP17A1 has been modelled using computational chemistry in order to understand the information about naturally occurring mutations and to provide a rational basis for designing alterations in the enzyme that probe functional domains of the protein (Auchus and Miller, 1999). The crystal structure of human CYP17A1, in complex with inhibitors abiraterone and TOK-001, has also been determined clarifying previous misconceptions regarding CYP17A1 structure (Nahri *et al.*, 1986) as well as prog and prog substrate specificity (DeVore and Scott, 2012).

### 3.2 Structural domains of CYP17A1

The P450 moiety (P450BMP) of the distinct bacterial microsomal enzyme P450BM-3 has been crystallized (Boddupalli *et al.*, 1992) and its structure solved (Ravvichandran *et al.*, 1993). This distantly related bacterial P450 was the template for molecular modelling and the first 3D structure for human CYP17A1 was established in 1999, with protein data bank identification number (pdb-id) 2C17 (Auchus and Miller, 1999). Subsequently, the crystallization and structure of the more closely related mammalian P450, cytochrome P450 2C5 (CYP2C5) pdb-id 1DT6, has been determined and is also used as a template for structural modelling of other human and mammalian P450s (Williams *et al.*, 2000b).

Figure 3-1 shows the alignment of core elements of human CYP17A1 with bacterial P450 class 2 enzymes (P450BMP), the principle template upon which the human CYP17A1 structural model is based (Auchus and Miller, 1999). Residues that form part of core helices and sheets are shown in bold. The most conserved residues are underlined. Among various species CYP17A1, conserved residues include: Trp121 in the C-helix; catalytic Thr306 in the I-helix; the Glu359xxArg362 sequence in the K-helix and the heme-liganding Cys442 (Auchus and Miller, 1999).

							<b>A' Helix</b>			
<b>BMP</b>							MTIKEMPQPKTFGELKNCPLLNTD	<b>24</b>		
<b>C17</b>							MWELVALLLLTLAYLFWPKRRCPGAKYPKSLLSLPLVGSPLPFLPRHG	<b>47</b>		
	<b>A Helix</b>		<b>Sheet 1</b>		<b>B Helix</b>		<b>Sheet 1</b>			
<b>BMP</b>	KPVQALMKIADEL	G	EIFKFEA	PG	RVTRYL	S	SQRLIKEAC	DES	RFDKN L	<b>71</b>
<b>C17</b>	HMHNFFKLOKKY	G	PIYSVRM	GT	KTTVIV	G	HHQLAKEVLI	KKG	KDFSG R	<b>96</b>
	<b>B' Helix</b>		<b>C Helix</b>		<b>D Helix</b>					
<b>BMP</b>	SQALKFVRDFAG	DGLFTSWTH	<u>EKNW</u> KKAHNILL		PSFSQQAMK		<u>GYHAMM</u> VDIADVQLVQKWER			<b>132</b>
<b>C17</b>	PQMATLDIASNN	RKGIAPADS	<u>GAHW</u> QLHRRRLAMA		TFALFKDGDQK		<u>LEKIIC</u> QEISTLCDMLATH			<b>160</b>
	<b>Sheet 3</b>		<b>E Helix</b>		<b>F Helix</b>					
<b>BMP</b>	LNADE HIEV		PEDMTRLTLDITIGLCGF		NYRFNSFYRDQPH		<u>PFITSM</u> VRALDEAMNKLQR	ANP	DDP	<b>196</b>
<b>C17</b>	NGQ SIDI SF		<u>PV</u> FVAVTNVISLICF		NTSYKN	GD	<u>PELNVI</u> QNYNEGIIDNLSK	DSLVDLVPWLKIF		<b>224</b>
			<b>G Helix</b>		<b>H Helix</b>		<b>Loop/Sheet 5</b>			
<b>BMP</b>	AYDENKRQFQEDIKVMNDLVDKIIADRKAS	GEQSD	<u>LL</u> THML		NGK	DPE T	GEP L			<b>249</b>
<b>C17</b>	PNKTLEKLGKSHVKIRNDLLNKILENYKEK	FRSDSIT	<u>NML</u> DTL		MQAKMNSDNGNAGPDQDSELLS					<b>288</b>
			<b>I Helix</b>		<b>J Helix</b>					
<b>BMP</b>	DDENIRYQIITFLIAGHET	<u>T</u> SGLLSFALYFLVK			<u>NPHVL</u> QKAAEEAARVL		VDPVP			<b>303</b>
<b>C17</b>	DNHILTTIGDIFGAGVET	<u>T</u> TSVVKWTLAFLLE			<u>NPQV</u> KKLYEEIDQNV		GFSRT			<b>341</b>
	<b>J' Helix</b>		<b>K Helix</b>		<b>Sheets 1&amp;2</b>					
<b>BMP</b>	SYKQVKQ		LKYVGMVLN <u>EAL</u> RLW		PTA	<u>PAF</u> SLYA K	EDTVL GG	<u>EY</u> PLE K	GDELMVL	<b>356</b>
<b>C17</b>	PTISDRNR		LLLLEATIRE <u>VLR</u> LR		PVA	<u>PML</u> IPHK A	NVDSS IG	<u>EFA</u> VD K	GTEVIIN	<b>395</b>
	<b>K' Helix</b>		<b>Meander</b>		<b>Heme-Binding</b>					
<b>BMP</b>	IPQLH	RDKTIWG	<u>DD</u> VEEFRPER		FENPSAI	PQHAFK	<u>PFG</u> NGQRACI			<b>401</b>
<b>C17</b>	LWALE	HNEKEW	<u>HQ</u> PDQFMPER		FLNPAGTQLISPSVSYL		<u>PFG</u> AGPRSCI			<b>443</b>
	<b>L Helix</b>		<b>Sheet 3</b>		<b>Sheets 4&amp;3</b>					
<b>BMP</b>	GQQFALHEATLVLGMMLK	HFDPE	DHTNY	ELD	IKET L	TLKPE G	<u>FVV</u> KAKSK	KIPLG		<b>456</b>
<b>C17</b>	GEILARQELFLIMAWLLQ	RFDLE	VPDQGQLPSLE		GIFK V	<u>VFL</u> ID S	<u>FKV</u> KIKVR	QAWRE		<b>501</b>

Figure 3-1: Alignments of core elements of human CYP17A1 (C17) and P450BMP (BMP). Residues forming part of core structures are shown in boldface type and the conserved residues are underlined (reproduced from Auchus and Miller, 1999).

Recently, the first X-ray crystal structures of CYP17A1 have been obtained (DeVore and Scott, 2012), in complex with two CYP17A1 inhibitors, abiraterone or TOK-001. Both inhibitors are involved in treatments of prostate- and or breast cancer (Attard *et al.*, 2008) and bind with heme Fe, which orients the structure above the heme plane at an angle of 60°, as well as being positioned against the central I helix. Asp202 (F helix) interacts with the  $\beta$ -hydroxyl group of C3 (3 $\beta$ -OH) and differs from the predicted by homology models and from steroids in other P450 enzymes with identified structures.

The global structure of CYP17A1 provides the groundwork for understanding mutations found in patients with steroidogenic diseases, the active site exposes multiple spatial arrangements and hydrogen bond characteristics, which justify for a superior understanding of the enzyme's dual catalytic potential.

Human CYP17A1 protein was engineered to exhibit a mutation that eliminated the N-terminal transmembrane helix (truncated) and was subsequently expressed in *E. coli*. CYP17A1 bound with either abiraterone or TOK-001, resulted in a type II difference spectra, which is consistent with the binding of nitrogen to the heme Fe with dissociation constant ( $K_d$ ) values of <100 nM.

CYP17A1 bound to prog or preg results in a type I substrate-induced difference spectra, which is indicative of the ligand displacing water from the heme. This modified human CYP17A1 bound preg more tightly than prog, with  $K_d$  values of <100 nM and 229 nM, respectively. Full-length CYP17A1 has a 3-fold higher  $K_m$  (11.4  $\mu$ M) compared to the truncated form ( $K_m$  3.7  $\mu$ M).

Although inhibitors reside in the majority of the roofed active site, the void extends beyond these ligands in several directions. The 3 $\beta$ -OH group of the inhibitors is the most prominent extension of the active site cavity, spanning the top of the I helix and along the underside of the F and G helices. The cavity is lined by mostly hydrophobic residues (Ile198, Leu243 and Phe300), although several polar residues (Tyr201, Asn202 and Arg239) of the F and G helices, border at the top of the cavity and interact with- or are located near water molecules. A single direct hydrogen bond exists between the inhibitors and CYP17A1 and it forms part of a larger hydrogen bond network linking with residues Asn202, Glu305, Arg239, the backbone carbonyl of Gly297, conserved water molecules and for some proteins, Tyr201 (DeVore and Scott, 2012).

These interactions strongly resemble interactions conserved in steroid hormone receptors, where in each deep receptor pockets, the 3 $\beta$ -OH group of steroids forms hydrogen bonds with Gln or glutamate, Arg and often a conserved water molecule. These interactions are vital for ligand recognition by hormone receptors and may also contribute to CYP17A1 selectivity for preg, prog and their 17 $\alpha$ -hydroxy counterparts. When preg and prog were substituted into the CYP17A1-abiraterone structure model (see Figure 3-2), prog maintained the hydrogen bond with residue Asn202. The distances from C17 and C16 to the catalytic heme oxygen were 3.7Å and 3.9Å, respectively, which was consistent with the data regarding the major 17OH- and minor 16OHprog metabolites (DeVore and Scott, 2012).

Aromatase (Ghosh *et al.*, 2009), P450scc (Mast *et al.*, 2011) and cholesterol 24-hydroxylase (CYP46A1) (Mast *et al.*, 2008) proteins maintain the characteristic folding of the cytochrome P450 structure and CYP17A1 can be compared to these reported steroid complex structures. However, aromatase, P450scc and CYP46A1 orientates steroid substrates in an opposite direction, as was



determined for CYP17A1, so that the steroid ligands are positioned over the K–L loop directed towards the B1 sheet instead of oriented towards helices F and G as with CYP17A1 (see Figure 3-2).

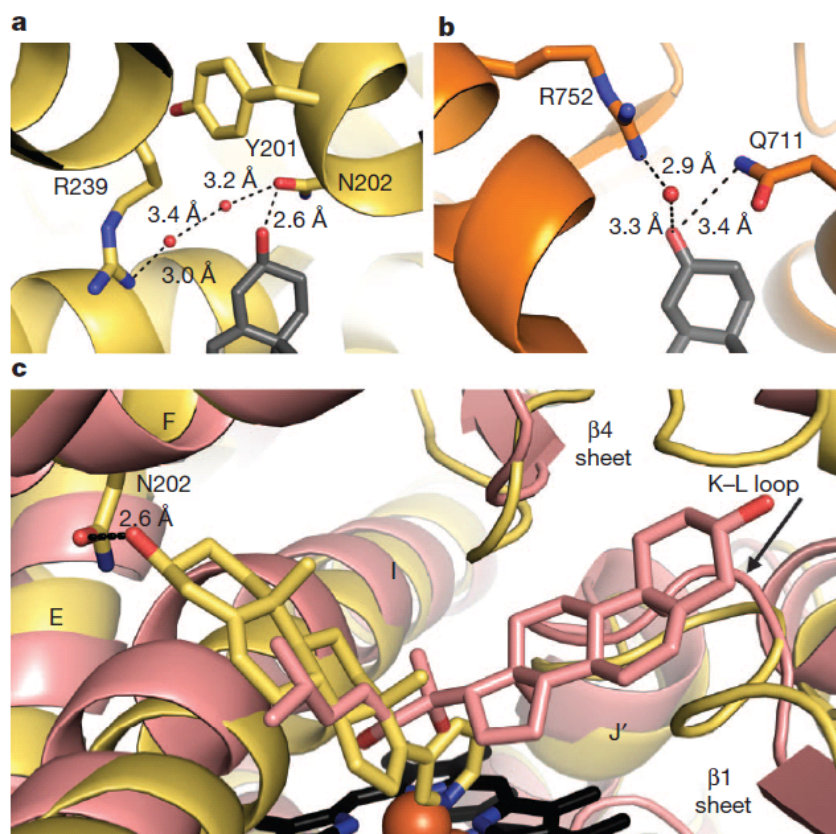


Figure 3-2: CYP17A1 structure compared to the AR and P450sc. (a) Hydrogen bond network near 3β-hydroxyl group of abiraterone involving Asn202, Arg239 and water. (b) The AR (pdb-id [3L3X](#)) has a similar hydrogen bond network with Arg752 and Glu711 with several waters interacting with DHT ketone. (c) The structure of CYP17A1, bound with abiraterone (yellow), superimposed on CYP11A1 structure with 20,22-dihydrocholesterol (pink). Different steroid orientations are observed for both structures (reproduced from DeVore and Scott, 2012).

### 3.2.1 Heme and its binding site

Heme is a chemical compound and a prosthetic group on numerous proteins permitting their cellular functions. The basic structure of heme consists of a ferrous ( $\text{Fe}^{++}$ ) ion contained in the center of a heterocyclic organic porphyrin ring, which constitutes up to four pyrrole groups joined together by methane bridges (Voet and Voet, 2004). Various types of heme exist, differing by their side groups. However, most forms are derived from heme *b* (Figure 3-3), where each pyrrole ring has one methyl group side-chain; two of the pyrrole rings have a vinyl group side-chain, while the other two rings have a propionic acid side-chain (propionate group). Many porphyrin-containing metalloproteins (including



cytochromes) have heme as their prosthetic group and are thus known as hemeproteins. In addition to the chemical properties of their environment, each hemeprotein has a precise amino acid sequence and structure that coordinates which heme it associates with, if it can associate and how.

The heme has four covalent bonds to nitrogen counterparts of pyrrole rings with  $p$  electrons contributing to its stability when the Fe is attached to the heme. The 5<sup>th</sup> available ligand of the Fe molecule is occupied by the thiolate side-chain of a cysteine residue in microsomal cytochrome P450s and the 6<sup>th</sup> site is occupied by dioxygen (Shimizu *et al.*, 1988). During electron transfer in P450 catalytic reactions, the Fe molecule cycles between  $\text{Fe}^{++}$  and  $\text{Fe}^{+++}$  states as it activates dioxygen for hydroxylation reactions.

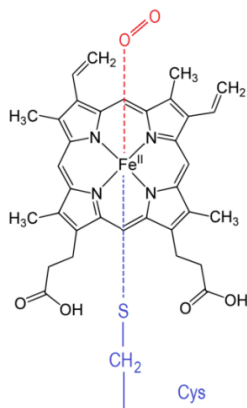


Figure 3-3: Chemical structure of heme *b* ( $\text{C}_{34}\text{H}_{32}\text{O}_4\text{N}_4\text{Fe}$ ). In all cytochrome P450s, the 5<sup>th</sup> and 6<sup>th</sup> ligands of the central Fe molecule in heme *b* are occupied by the thiolate side-chain of a cysteine residue (*blue*) and dioxygen (*red*), respectively (reproduced from Shimizu *et al.*, 1988).

The heme-binding domain of CYP17A1 is highly conserved among species and consists of residues Pro434-Ile443 with the Cys442 residue functioning as the axial ligand of the heme Fe. Residue Arg440 forms a hydrogen bond with the propionate moiety and is a constant observation in all P450 structures (Ravichandran *et al.*, 1993). The amino acid substitution Arg440Cys causes complete 17 $\alpha$ -hydroxylase deficiency in P450s (Fardella *et al.*, 1994). The substitution His373Leu renders the P450 enzyme incapable of binding with heme and causes 17 $\alpha$ -hydroxylase deficiency (Monno *et al.*, 1993). Residue His373 does not interact with heme but forms a hydrogen bond with the carboxylate group of residue Glu391 in the neighbouring strand of a  $\beta$ -sheet near the membrane attachment-site. Therefore, His373Leu appears to alter the general structure of the P450, which indirectly forbids heme binding (Monno *et al.*, 1993).

### 3.2.2 The substrate-binding pocket

Human CYP17A1 does not possess a bi-lobed substrate binding pocket, as was previously predicted by CYP17A1 models based on bacterial P450s (Nahri *et al.*, 1986; Lin *et al.*, 1994), which was corroborated by DeVore and Scott (DeVore and Scott, 2012). Miller and Auchus (1999) define this pocket by: the heme-group, which forms the floor of the pocket; the I-helix, which runs along the edge of the heme ring; strands 4 and 5 of  $\beta$ -sheet 1, which is opposite the I-helix; residue Ile112, which is in the B'-C'-loop on the one side; the loop after the K-helix on the other side and lastly, by residues Val482 and Val483, which forms a turn in  $\beta$ -sheet 3 forming the top of the substrate binding pocket (Auchus and Miller, 1999). Multiple residue interactions are found to limit the extent of the pocket above the heme. Figure 3-4 shows the structural model of human CYP17A1 binding pocket occupied with a steroid substrate.

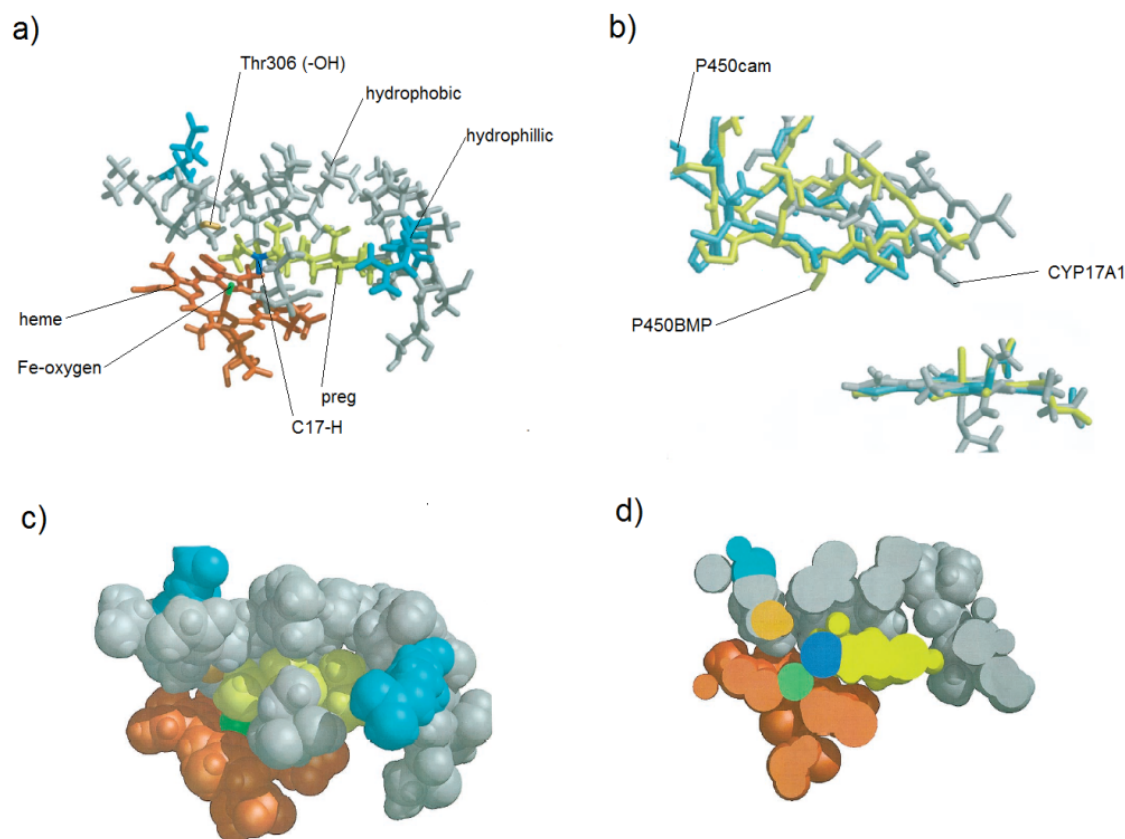


Figure 3-4: A model of steroid-bound CYP17A1 (pdb-id [2C17](#)). Heme is shown in *red*; the iron-oxy in *green*; Thr306 (-OH) group in *orange*; CYP17A1 hydrophobic residues in *gray* and hydrophilic residues in *cyan*. The steroid (preg) is shown in *yellow* with C17 and its attached hydrogen (C17-H) in *blue*. (a) A stick diagram shows that the entire substrate-binding pocket of CYP17A1 is defined by hydrophobic residues and that the two hydrophilic residues do not contribute to the surface boundary of the pocket. (b) A comparison of crystal structures for P450cam (*cyan*), P450BMP (*yellow*) and CYP17A1 (*gray*) show that  $\beta$ -sheet 3 extends closer towards the heme and occupies more steroid-binding space in P450BMP and CYP17A1, than in P450cam, thus restricting the size of the substrate-binding pocket. Although modelling core elements initially positioned the  $\alpha$ -carbons of CYP17A1 in the same locations as the corresponding  $\alpha$ -carbons of P450BMP, the thermo- and molecular dynamic procedures shifted the CYP17A1 backbone significantly further restricting the size of the CYP17A1 substrate-binding pocket. (c) The conic space-filling diagram shows that although the steroid fills virtually the entire substrate-binding pocket, there is space above the steroid for water molecules. (d) A sagittal sectional view of the conic diagram (through Thr306, Fe-oxygen and C17 of the steroid), shows that three key components of the  $17\alpha$ -hydroxylase reaction are in close proximity (reproduced from Auchus and Miller, 1999).

Previously, it was also proposed that the substrate was positioned perpendicularly to the heme (Lin *et al.*, 1994). However, the smaller substrate-binding pocket found in the human CYP17A1 model prevents this binding orientation and rather limits the substrate to a position where the planes of the steroid and heme ring are more or less parallel to one another (Auchus and Miller, 1999). This orientation restricts steroid substrates to a single orientation, which places C17 directly above the heme Fe. Additionally, this orientation facilitates hydrophobic interactions between the C18 and C19 methyl groups of the steroids and the side-chains of CYP17A1 residues Pro368, Val482, Val483 and Ala302.

### 3.3 Key residues involved in CYP17A1 activity

Residues Ile344, Ser345, Asp346, Arg347, Asn348 and Arg358 are conserved in most species (particularly in baboon, bovine, chimpanzee, angora goat, macaques, porcine and sheep) with the exceptions of rat and mouse (see Addendum A). Lys89 is conserved in all species, including rat and mouse, and together with Arg347 and Arg358 are highlighted specifically to be involved with the redox binding proteins. These residues form positive charges on the proximal surface of the enzyme. Neutralizing these charges impairs 17,20-lyase activity without reduction in 17 $\alpha$ -hydroxylase activity (Auchus *et al.*, 2000).

Substrate preferences for CYP17A1 have been shown to be species-specific. The  $\Delta$ 5 pathway is favoured in human, bovine, and pig adrenal glands and testes (Miller, 1988) and the  $\Delta$ 4 pathway is prevalent in rat testis (Fevold *et al.*, 1989). Substrate preferences are also evident in transfected non-steroidogenic COS-1 cell line, where the source of the differential  $\Delta$ 4 and  $\Delta$ 5 activity has been identified at the level of the lyase activity (Zuber *et al.*, 1986). Bovine CYP17A1 has a specific reduction in the  $\Delta$ 4-lyase activity, but not expressed rat CYP17A1. The initial reactions catalyzed by human/bovine or rat species display comparable  $\Delta$ 4 and  $\Delta$ 5 17 $\alpha$ -hydroxylase activities. Therefore, expression of human/bovine and rat CYP17A1 in non-steroidogenic cell lines (Zuber *et al.*, 1988), can be distinguished on the basis of changes in  $\Delta$ 4-supported lyase activity (Fevold *et al.*, 1989), and these changes may be related to the species-specific amino acid substitutions within the conserved domain (Chung *et al.*, 1987).

Arg346 forms part of a putative lyase domain (residues 343-348) in rat CYP17A1 and is essential for lyase activity (Picardo-Leonard and Miller, 1988; Kitamura *et al.*, 1991). Site-directed mutagenesis generated rat CYP17A1 mutant, with substituted residues identical to bovine residues at those positions, to determine if the bovine-specific reduction in  $\Delta$ 4 lyase activity results from changes in the primary sequence that may influence substrate interaction and or specific transport of the  $\Delta$ 4 intermediate out of the active-site domain. Expression and analysis of the  $\Delta$ 4-lyase activity of mutant rat CYP17A1 in COS-1 cells, revealed that rat mutation Phe343Ile resulted in a reduction in  $\Delta$ 4-lyase activity to levels approaching the range of the  $\Delta$ 5-reaction (Koh *et al.*, 1993). Rat CYP17A1 with substitutions Asn344Ser, Ser347Asn and His348Arg, did not exhibit changes in  $\Delta$ 4-lyase activity or in  $\Delta$ 4- and  $\Delta$ 5 17 $\alpha$ -hydroxylase activities. Therefore, rat CYP17A1 with substitution at position 343 with bovine amino acid residue Ile, imitates the reduced activity of the bovine CYP17A1  $\Delta$ 4 lyase reaction.

The rat Phe343 mutant exhibited a low level lyase activity that did not differentiate between  $\Delta 4$ - and  $\Delta 5$  substrates. Results suggest that the occurrence of Phe343 enhances the  $\Delta 4$  lyase activity potentially through stabilization of  $\Delta 4$  specific interface and facilitating either the  $\Delta 4$  lyase reaction or  $\Delta 4$  substrate binding (Koh *et al.*, 1993).

Figure 3-5 shows known mutations in the redox partner binding site of human CYP17A1. These mutations cause the characteristic loss in 17,20-lyase activity by neutralizing positively charged residues. Phe417 is not exposed to the protein surface but lies behind a loop between the meander region and the heme binding region (Gupta *et al.*, 2001).

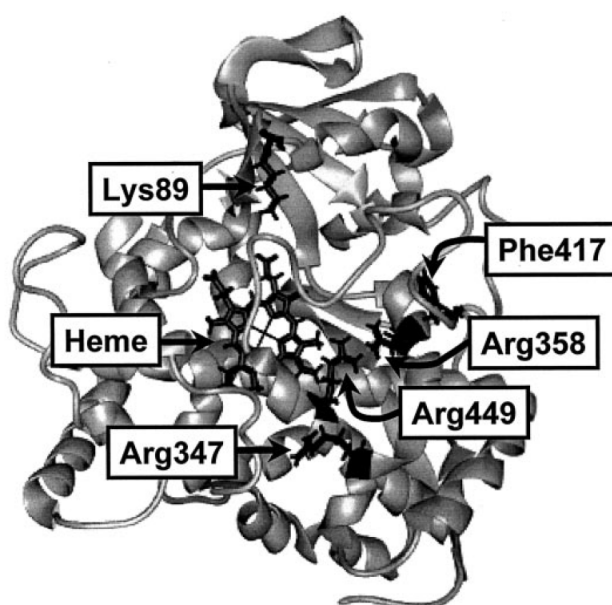


Figure 3-5: Mutations in the redox partner binding pocket of human CYP17A1, causes the loss in 17,20-lyase activity (Gupta *et al.*, 2001).

Mutations that specifically impair the 17,20-lyase activity of CYP17A1 are mostly informative for structure-function studies of this protein and may also give insight into the development of adrenarche (Gupta *et al.*, 2001). Geller *et al.* (1997) identified patients who were homozygous for the mutations Arg347His and Arg358Gln that neutralized single positive charges in the redox partner binding site (Geller *et al.*, 1997). These two mutations are associated with isolated 17,20-lyase deficiency. They dramatically impair lyase activity in the presence or absence of CYB5, whilst 17 $\alpha$ -hydroxylase activity remained preserved (Geller *et al.*, 1997). Gupta *et al.* (2001), using molecular models and biochemical studies, described these mutations as impairing the interactions of CYP17A1 with redox partners CPR

and CYB5. They also concluded that neutralizing key positive charges in the redox partner binding site can cause the isolated loss of 17,20-lyase activity (Gupta *et al.*, 2001). Lys89Asn (Auchus and Miller, 1999) and Arg449Ala (Lee-Robichaud *et al.*, 1999) are two other mutations that map to the redox binding site but cause a reduction in 17,20-lyase activity.

Phe417Cys has been considered to cause 17,20-lyase deficiency, however it was suggested that this mutation does not lie within the redox partner binding site but adjacent to it. Amongst other possible mechanisms, this mutation may introduce changes to the structure of the protein that are propagated to the redox partner binding site. Gupta *et al.* (2001) found that although this mutant protein could be expressed, it was not active and the heme did not bind correctly nor did it exhibit 17 $\alpha$ -hydroxylase activity (Gupta *et al.*, 2001). Two prevalent CYP17A1 mutations in humans which cause 17 $\alpha$ -hydroxylase deficiency are Trp406Arg and Arg362Cys (Costa-Santos *et al.*, 2004).

The human CYP17A1 amino acid sequence has 66.7% sequence identity with the porcine protein. cDNA comparisons indicated that a central region (consisting of amino acids 160-268) is hypervariable in human, porcine and bovine proteins (Chung *et al.*, 1987). Adrenal and testicular CYP17A1 mRNA are mostly indistinguishable (Chung *et al.*, 1987), although minor differences have been reported in porcine adrenal or testicular CYP17A1 (Nakajin *et al.*, 1984). These proteins were defined by molecular weight, amino acid composition and N-terminal amino acid sequence but were enzymologically and immunologically indistinguishable. This raised the possibility of tissue-specific isozymes, which to date, remains to be confirmed. Porcine adrenal CYP17A1 amino acid sequence differs from porcine testes CYP17A1 at a single position. Adrenal cDNA encodes a His407 residue (GenBank accession number [AAA84419.1](#)) and testes cDNA encodes a Leu407 residue (GenBank accession number [NP\\_999593.1](#)).

The aim of this study is to compare the 17 $\alpha$ -hydroxylase/17,20-lyase and 16 $\alpha$ -hydroxylase activities of wt porcine CYP17A1 and to determine the influence of targeted amino acid substitutions on the catalytic activity of the wt. Adrenal porcine CYP17A1 cDNA (wt pCYP17A1) was obtained from collaborators at Edinburgh University, Scotland. Direct sequencing analysis of this clone revealed an amino acid sequence different to that obtained from porcine CYP17A1, cloned from cDNA isolated from porcine testicular cDNA (Conley *et al.*, 1992). Sequence analysis of wt pCYP17A1 showed residues Val40 and His407 (designated -VH), whereas CYP17A1 purified from the porcine adrenal has Leu40 and His407 (designated -LH). CYP17A1 cloned from porcine testes has residues Leu40 and Leu407 (designated -LL). Although these differences may be due to PCR artifacts, it is interesting to

note that Chung *et al.* (1987) isolated and purified CYP17A1 protein from porcine adrenal tissue and subsequent amino acid analysis identified a His residue at position 407 (Chung *et al.*, 1987). Human CYP17A1 (Picardo-Leonard and Miller, 1987) also has a Leu residue at position 40 and a His residue at position 407. Employing site-directed mutagenesis using wt pCYP17A1-VH as the template, single mutants (pCYP17A1-LH and -VL) and a double mutant (pCYP17A1-LL) at amino acid positions 40 and 407, respectively, were prepared. This study also investigated the 16 $\alpha$ -hydroxylase activity characteristic of human CYP17A1, which has an Ala residue at position 105 while wt pCYP17A1-VH has a Leu residue. A wt pCYP17A1 mutant construct (designated -LA), was thus prepared to investigate the 16 $\alpha$ -hydroxylase activity, in which Leu105 was mutated to an Ala residue. All mutations were confirmed by direct sequence analysis. Catalytic studies were conducted using prog as substrate in non-steroidogenic mammalian cells expressing the recombinant enzymes.

The integration of recombinant cDNA in plasmid vectors into specific cell lines is a very efficient method of examining enzyme function. Transformed (Simian Virus 40) African Green Monkey kidney fibroblast cells (COS-1) are non-steroidogenic cells and serve most useful in the investigation of enzyme activity with steroid substrate added to growth medium. COS-1 cells contain a single integrated copy of the complete early region of SV40 DNA and express the SV40 large tumour antigen, which enables transcription initiation at the SV40 origin site present in mammalian expression vectors such as pCMV5 and pcDNA3.2 (Gluzman, 1981; Strayer, 1996). This results in a high copy number of the vector in each transfected cell (Aruffo, 2001). Furthermore, COS-1 cells also have sufficient amounts of endogenous CPR to confer maximal 17 $\alpha$ -hydroxylase activity in CYP17A1 (Lin *et al.*, 1993), making these cells ideal for the investigation of the influence of Val40Leu, His407Leu and Leu105Ala substitutions on the catalytic activity of porcine CYP17A1.

### **3.4 Materials and methods**

#### **3.4.1 Plasmids**

Porcine pCMV5\_CYP17A1 (wt pCYP17A1-VH) plasmid (cloned via *Bam*HI in MCS) was a kind gift from Prof. Ian Mason of Edinburgh University, Scotland. pCI-neo (Promega) and angora CYP17A1 (aCYP17A1) plasmids were available in the laboratory and included as experimental control plasmids.



### 3.4.2 Reagents

<sup>3</sup>H-Prog was purchased from PerkinElmer Life Sciences (Boston, MA, USA). Prog, 17OHprog, 16OHprog, A4 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Chemical Company (St. Louis, MO, USA). COS-1 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). Penicillin-streptomycin and trypsin-EDTA were purchased from Gibco-BRL (Gaithersburg, MD, USA). Fetal calf serum and bacterial culture media were purchased from Highveld Biological (Lyndhurst, RSA). GeneJuice transfection reagent was purchased from Novagen (Darmstadt, Germany). DNA marker, *marker VI* (digested pBR328 DNA/*BglI* and pBR328 DNA/*HinfI*) was purchased from Roche Applied Sciences (Mannheim, Germany). Plasmid pCI-neo, DNA markers  $\lambda$ DNA/*EcoRI*+*HindIII* (digested *lambda* DNA/*EcoRI*+*HindIII*) and 1 kb DNA marker, ribonucleotide phosphates and Wizard® Plus SV Miniprep DNA Purification systems were purchased from Promega Biotech (Madison, WI, USA). Restriction endonucleases *BamHI*, *XhoI* and *EcoRI* were purchased from Fermentas (Burlington, Canada). Primers were purchased from Integrated DNA Technologies (Coralville, Iowa). Nucleobond® AX plasmid preparation kits were purchased from Machery-Nagel (Duran, Germany). Platinum Taq Polymerase PCR kits, SYBR®Safe DNA gel stain and GeneTailor™ Site-Directed Mutagenesis Systems were purchased from Invitrogen (Carlsbad, California). A Pierce BCA™ protein assay kit was purchased from Pierce Chemical (Rockford, IL, USA). All other chemicals were of the highest analytical grade and purchased from scientific supply houses.

### 3.4.3 Wt pCYP17A1-VH preparation and plasmid integrity

The plasmid construct was received blotted on filter paper. The circular centres, 3 mm x 3 mm, were cut out and resuspended in TRIS buffer (10 mM, pH 7.6), 50  $\mu$ l. Resuspended plasmid, 2  $\mu$ l, was added to freshly prepared *E. coli* JM109 competent cells, 200  $\mu$ l, prepared using the CaCl<sub>2</sub> (75 mM) method (Sambrook *et al.*, 1989), placed on ice for 30 min, heat shocked at 42°C for 90 sec and then placed on ice for 90 sec. SOC medium, 800  $\mu$ l, was added to the transformed cells after which the cells were incubated at 37°C for 1 hr at 225 rpm. Cells were plated on LB medium-agar containing ampicillin, 100  $\mu$ g/ml, and grown overnight at 37°C with plates placed upside-down.

LB medium, 5 ml, containing ampicillin, 100  $\mu$ g/ml, was inoculated with a single colony of transformed *E. coli* cells and incubated overnight at 37°C at 225 rpm. Plasmid DNA was isolated using



the Wizard® Plus SV Minipreps DNA Purification System, according to manufacturer's instructions (Promega). After precipitation, the plasmid DNA pellet was redissolved in nuclease-free water, 100 µl, and the plasmid yield was calculated by UV spectrophotometry, 260/280 nm.

Plasmid constructs were screened by restriction digestion analysis and positive clones were subsequently subjected to sequence analysis with primers listed in Table 3-1. All DNA sequencing was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster city, California).

Table 3-1: Primer sequences used in the direct DNA sequencing of wt pCYP17A1-VH. First left and right primer pair (LP1 and LP2) are complementary to the 5' and 3' ends of porcine CYP17A1 (GenBank accession number [NM\\_214428.1](#)), respectively. The second and third left and right primer pairs are complementary to the 5' and 3' ends of wt pCYP17A1-VH, respectively and sequence the remaining length of DNA.

Primer	Oligonucleotide sequence
LP1 (Sense)	5'-GGTCAATGCTATCCTGGATC-3'
RP1 (Antisense)	5'-CACTGGAGTGGCAACTTC-3'
LP2 (Sense)	5'-CTGCAGGCCATAGTGAAT-3'
RP2 (Antisense)	5'-ACTATGTTGGTCATCGCC-3'
LP3 (Sense)	5'-CACAGATGTCGTCGTCAA-3'
RP3 (Antisense)	5'-TGACGACGACATCTGTGT-3'

Plasmid DNA was subjected to single enzyme restriction digests in a reaction volume of 20 µl with nuclease-free water, including: appropriate single enzyme BSA-buffer (10X), 1X, 2 µl; plasmid DNA (1 µg/µl), 1 µl, and restriction enzyme *Bam*HI (10 U/µl), 5 U, 0.5 µl; *Xho*I (10 U/µl), 5 U, 0.5 µl; or *Eco*RI (10 U/µl), 5 U, 0.5 µl, respectively, for 1 hr at 37°C. Plasmid DNA was subjected to a double enzyme restriction digest in a reaction volume of 20 µl with nuclease-free water, including: Tango™ BSA-buffer (10X), 2X, 4 µl; plasmid DNA (1 µg/µl), 1 µl, and restriction enzymes *Eco*RI (10 U/µl), 10 U, 1 µl and *Xho*I (10 U/µl), 10 U, 1 µl for 1 hr at 37°C. The products were separated by 1% agarose gel electrophoresis at 55 V with 1 kb DNA marker and stained with SYBR®Safe for UV visualization.

#### 3.4.4 Site-directed mutagenesis

Site-directed mutagenesis of porcine CYP17A1 was carried out using the GeneTailor™ site-directed mutagenesis system (Invitrogen) according to manufactures instructions, using the primers listed in

Table 3-2. The wt pCYP17A1-VH clone was used as the template, which contains the cDNA encoding porcine CYP17A1.

Table 3-2: Primer sequences used in the site directed mutagenesis of wt pCYP17A1-VH. Left and right mutagenesis primer pairs (V40L-LP, V40L-RP, H407L-LP, H407L-RP, L105A-LP and L105A-RP) are complementary to the 5' and 3' ends of wt pCYP17A1-VH, respectively and incorporate the appropriate nucleotide substitutions encoding the desired amino acid change. The codons for the changed amino acid appear in bold characters and the substituted nucleotide is underlined.

Primer	Oligonucleotide sequence
V40L-LP (Sense)	5'- CTGCCCCTGGTGGGCAGC <u>C</u> TGCCATTCCTA-3'
V40L-RP (Antisense)	5'- GCTGCCACCCACGGGCAGGACTGGGAGACT-3'
H407L-LP (Sense)	5'- TCACAATGAGAAGGAGTGGC <u>T</u> CCGGCCCCGAC-3'
H407L-RP (Antisense)	5'-GCCACTCCTTCTCATTGTGATGCAGTGCCC-3'
L105A-LP (Sense)	5'-AGAGTGATGACTCTAGACATC <u>GCG</u> TCAGACAACC-3'
L105A-RP (Antisense)	5'-GATGTCTAGAGTCATCACTCTGGGCCGCC-3'

The DNA methylation reaction was carried out in a final volume of 16  $\mu$ l using sterile RNA/DNA free water including: methylation buffer (10X), 1X, 1.6  $\mu$ l; SAM (10X), 1X, 1.6  $\mu$ l; plasmid DNA template (100 ng), 1  $\mu$ l, and DNA methylase (4 U/ $\mu$ l), 4 U, 1  $\mu$ l. This reaction mixture was placed in a water bath at 37°C for 1 hr and then on ice. The mutagenesis reaction was made up to a volume of 50  $\mu$ l using RNA/DNA-free sterile water: High Fidelity buffer (10X), 1X, 5  $\mu$ l; dNTP (20 mM), 10 mM, 0.75  $\mu$ l; MgSO<sub>4</sub> (50 mM), 1 mM, 1  $\mu$ l; left and right mutagenic primers (10  $\mu$ M), 300 nM, 1.5  $\mu$ l each; methylated DNA (12.5-31.25 ng), 3  $\mu$ l, and Platinum Taq Polymerase (5 U/ $\mu$ l), 2.5 U, 0.5  $\mu$ l. PCR amplification was performed as follows: 94°C for 2.5 min, followed by 20 cycles 94°C for 30 sec, 55°C for 30 sec and 68 °C for 7 min, with a final elongation step at 68 °C for 7 min. The amplified mutant DNA fragments were separated on a 1% agarose gel by electrophoresis at 65 V with DNA marker  $\lambda$ DNA/*EcoRI* and *HindIII*. The gel was stained with ethidium bromide for visualization.

PCR product, 2  $\mu$ l, was added to One-Shot<sup>®</sup> MAX Efficiency<sup>®</sup> DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> *E. coli* competent cells, 50  $\mu$ l, on ice. The transformation reaction was placed in a water bath at 42°C for 30 sec, followed by 1 min incubation on ice. Room temperature SOC medium, 200  $\mu$ l, was added to the cells after which the cells were incubated at 37° for 1 hr. Cells were plated on LB medium-agar containing ampicillin, 100  $\mu$ g/ml, and grown overnight at 37°C with plates placed upside-down.

LB medium was inoculated with single colonies and incubated for 12-14 hr after which plasmid DNA was purified as previously described in section 3.4.3.

The desired mutations, Val40Leu, His407Leu and Leu105Ala were confirmed by direct DNA sequence analysis with primers listed in Table 3-1. Wt and mutant cDNA, at positions 40 and 407, will be referred to by single letter abbreviations of the amino acids they possess at those respective positions, listed in Table 3-3. Wt mutant containing Ala105, pCYP17A1-L105A, will be referred to as pCYP17A1-LA, which shows the single letter abbreviation for the amino acids substituted at position 105.

Table 3-3: Wt mutants are designated names based on one-letter abbreviations of amino acids present at respective positions and in comparison with the wt pCYP17A1-VH sequence. Double letter designations (-LH, -VL and -LL) refer to amino acids present at position 40 and 407, respectively and -LA refers to the amino acids substituted at position 105.

wt/mutant	Substitutions	Positions of amino acid differences			Designated name
wt		Val40	Leu105	His407	wt pCYP17A1-VH
wt mutant	V40L	<b><u>Leu40</u></b>	Leu105	His407	pCYP17A1- <b><u>LH</u></b>
wt mutant	H407L	Val40	Leu105	<b><u>Leu407</u></b>	pCYP17A1- <b><u>VL</u></b>
wt mutant	V40L; H407L	<b><u>Leu40</u></b>	Leu105	<b><u>Leu407</u></b>	pCYP17A1- <b><u>LL</u></b>
wt mutant	L105A	Val40	<b><u>Ala105</u></b>	His407	pCYP17A1-LA

### 3.4.5 Plasmid preparation

LB medium, 100 ml, containing ampicillin, 100 µg/ml, was inoculated with freezer stocks of plasmid-containing *E. coli* cells, 100 µl, and incubated overnight at 37°C at 225 rpm. The plasmid DNA was isolated using the Nucleobond® AX100 DNA Isolation Kit, according to manufacturers' instruction (Machery-Nagal). After precipitation, the plasmid DNA pellet was redissolved in deionized water, 200 µl, and the plasmid yield was calculated by UV spectrophotometry, 260/280 nm.

### 3.4.6 Conversion assays in non-steroidogenic mammalian COS-1 cells

COS-1 cells were grown to confluence in 100 mm culture dishes at 37°C and 5% CO<sub>2</sub> in DMEM, 10 ml; supplemented with 0.9 g/L glucose, 0.12% NaHCO<sub>3</sub>, 10% fetal calf serum and 1% penicillin–streptomycin (stock containing 10 000 U/ml penicillin and 10 000 µg/ml streptomycin). Twenty-four hours prior to transfection, cells were plated into 12-well culture dishes with each well containing 1×10<sup>5</sup> cells (1 ml/well). Cells were transfected by adding transfection reaction mix, 50 µl DMEM without fetal calf serum or penicillin–streptomycin, which contained plasmid DNA, 0.5 µg, and GeneJuice transfection reagent, 1.5 µl. Control transfection reactions were performed using the

mammalian expression vector pCI-neo (containing no cDNA insert) and a positive control plasmid, angora CYP17A1. The cells were incubated for 72 hr, after which enzyme activity was assayed by adding steroid substrate 1  $\mu$ M prog with or without  $^3$ H-prog (100 000 cpm/50  $\mu$ l) to fresh growth medium. Aliquots of 50  $\mu$ l or 500  $\mu$ l were removed at specific time intervals to assay substrate conversion and were analysed by high performance liquid chromatography (HPLC) or ultra performance liquid chromatography-mass spectrophotometry (UPLC-MS), respectively. Deionised water, 450  $\mu$ l was added to the tritiated samples (50  $\mu$ l) after which steroid metabolites were extracted in dichloromethane, 5 ml, for analyses by HPLC. Non-tritiated samples (500  $\mu$ L) were extracted in dichloromethane, 5 ml and steroid metabolites for analyses by UPLC. The dichloromethane phase was evaporated under a stream of nitrogen. Dried steroid residue was redissolved in methanol, 120  $\mu$ l, prior to analysis. After the last time-point, cells were washed with and collected in phosphate buffer (0.1 M, pH 7.4) and sonicated prior to determining the protein content by the Pierce BCA method (Pierce Chemical) according to the manufacturers' instructions.

#### **3.4.7 Separation and quantification of steroid metabolites**

HPLC steroid separation was performed on a Waters (Milford, MA, USA) high performance liquid chromatograph coupled to a WISP™ automatic injector (Waters) and a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL). Prog, 17OHprog and A4 were separated on a Novapak® C18 column at a flow rate of 1 ml/min. The mobile phase consisted of solvent A (35% water and 65% methanol) and solvent B (100% methanol). The column was equilibrated for 10 min with solvent A, followed by a linear gradient from 100% solvent A to 100% solvent B in 5 min, after which an isocratic elution of solvent B ran for 2 min.

UPLC-MS steroid separation was performed using a Waters UPLC BEH C18 (2.1 mm  $\times$  100 mm, 1.7  $\mu$ m) column (ACQUITY UPLC, Waters, Milford, USA) to separate and quantify prog, 17OHprog, 16OHprog and A4. The mobile phases consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B). Steroids were eluted at a flow rate of 0.35 ml/min, starting with the mobile phase 85% solvent A and 15% solvent B for 3.5 min, followed by 20% solvent A and 80% solvent B for 2.1 min, followed by 100% solvent B for 1 min and finally 85% solvent A and 15% solvent B for 2 min. An API Quattro Micro tandem mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. An Ion Sabre probe (Waters, Milford, USA) was used for the APCI interface in positive mode. The corona pin was set to 7  $\mu$ A, the cone voltage to 30 V and APCI probe

temperature was 100°C. All other settings were optimized to obtain the strongest signal possible. Data was collected with the MassLynx 4.0 software program.

### 3.5 Results

#### 3.5.1 Plasmid yield, integrity and sequence

Table 3-4 shows the absorbance values obtained from UV spectrophotometry analysis of isolated wt and mutant plasmid DNA samples. Concentration and purity of plasmid DNA was calculated using absorption values at 260 and 280 nm. OD values ranged between 0.1 and 0.8 for optimal measurement. Double strand plasmid DNA yield and concentration was calculated for 200 µl (wt) and 50 µl (mutant) plasmid DNA samples with 1 A<sub>260</sub> Unit of dsDNA = 50 µg/ml H<sub>2</sub>O and purity was determined by  $A_{260}/A_{280} \geq 1.6$ .

Table 3-4: Absorbance values of wt pCYP17A1-VH and mutant cDNA plasmids.

Isolated DNA	Absorbance values		Dilution	Concentration	dsDNA	Purity
	260 nm	280 nm	Factor	(µg/µl)	(µg)	260/280
wt pCYP17A1-VH	0.65	0.40	150	4.88	975.0	1.65
pCYP17A1-LH	0.70	0.43	8	0.28	14.0	1.64
pCYP17A1-VL	0.42	0.22	10	0.21	10.5	1.93
pCYP17A1-LL	0.57	0.29	8	0.23	11.5	1.95
pCYP17A1-LA	0.73	0.37	20	0.73	36.5	1.98

Direct sequence analysis of wt pCYP17A1-VH and mutants showed a total of 1786 bp. Table 3-5 shows plasmid DNA sequence recognition sites for restriction enzymes *EcoRI* (5'-G/AATTC-3'), *XhoI* (5'-C/TCGAG-3') and *BamHI* (5'-G/GATCC-3').

Table 3-5: Restriction enzyme recognition sites in (a) expression vector, pCMV5; (b) linear porcine CYP17A1 encoding cDNA sequence and (c) vector containing porcine CYP17A1 cDNA ligated into MCS in pCMV5 with *Bam*HI (wt pCYP17A1-VH). Restriction enzymes have one recognition site per sequence; except *Xho*I, which has only one recognition site in pCMV5 and *Eco*RI, which has two recognition sites in wt pCYP17A1-VH.

Sequence	Total size (bp)	<i>Eco</i> RI bp position	<i>Xho</i> I bp position	<i>Bam</i> HI bp position
(a) pCMV5 (circular)	4657	920	1973	980
(b) porcine CYP17A1 (linear)	1786	371		670
(c) wt pCYP17A1-VH (circular)	6443	920; 1351	3759	1650

Figure 3-6 shows the calculated fragment sizes of DNA products as a result of single/double endonuclease digestion of wt pCYP17A1-VH. Wt pCYP17A1-VH digested with *Bam*HI or *Xho*I, digesting at one site each, both result in linear DNA of 6443 bp. Wt pCYP17A1-VH digested with *Eco*RI (Figure 3-6a), digesting at two positions, results in DNA lengths of 431 bp and 6012 bp, respectively. Wt pCYP17A1-VH digested with *Eco*RI and *Xho*I (Figure 3-6b), digesting at three sites, results in DNA lengths of 431, 2408 and 3604 bp, respectively.

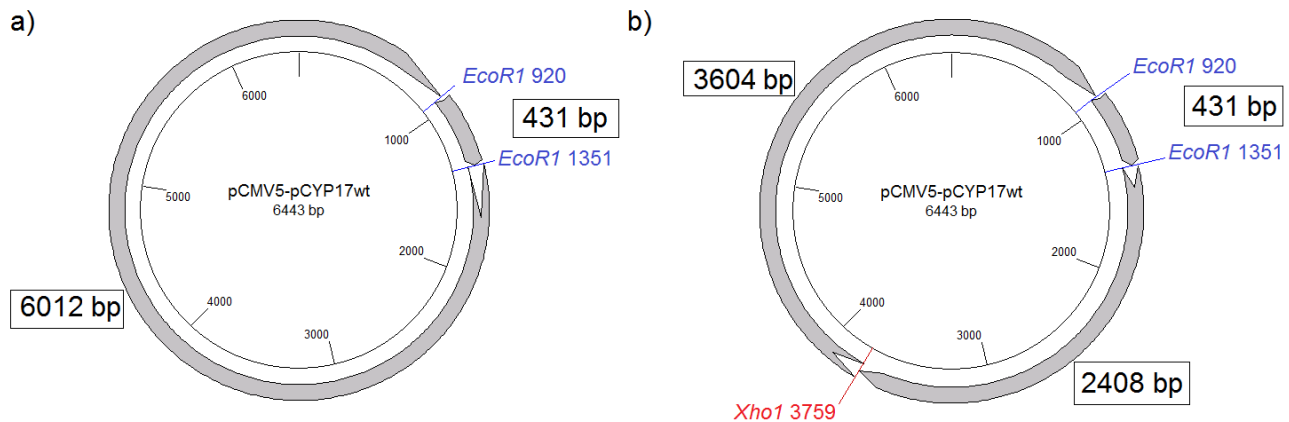


Figure 3-6: Calculated DNA fragment sizes after wt pCYP17A1-VH plasmid (pCMV5\_pCYP17A1wt) was (a) digested using endonuclease *Eco*RI and (b) double digested using endonucleases *Eco*RI and *Xho*I.

Figure 3-7 shows the analyses of DNA constructs using gel electrophoresis on a 1% agarose. Undigested plasmid wt pCYP17A1-VH (a); DNA products of plasmid enzyme digestion (b), as well as wt mutant PCR products (c-f) are shown in Figure 3-7. The  $\lambda$ DNA/*Eco*RI+*Hind*III marker ranges in base pair lengths of DNA fragments from 564 bp to 21 227 bp and the 1 kb marker ranges from 250 bp to 10 000 bp. Wt pCYP17A1-VH plasmid DNA comprise mostly supercoiled conformations and to a lesser extent, nicked (Figure 3-7a).

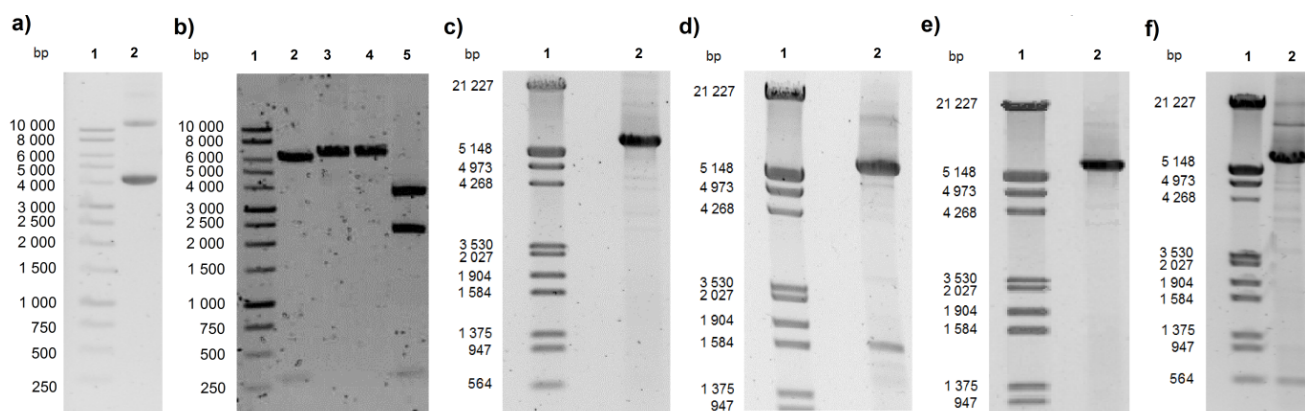


Figure 3-7: Images of undigested and digested wt pCYP17A1-VH plasmid and site-directed mutagenic constructs. (a) Lane 1 shows 1 kb marker and lane 2 shows undigested wt pCYP17A1-VH plasmid DNA. (b) Lane 1 shows 1 kb marker and wt pCYP17A1-VH single digested by *EcoRI* (lane 2), *XhoI* (lane 3), *BamHI* (lane 4) and a double digested by *EcoRI* and *XhoI* (lane 5). (c-f) First lanes show λDNA/*EcoRI*+*HindIII* marker. Lanes 2 show mutagenic PCR products: pCYP17A1-LH (c); pCYP17A1-VL (d); pCYP17A1-LL (e) and pCYP17A1-LA (f). DNA was separated by electrophoresis using a 1% agarose gel, subsequently stained with SYBR<sup>®</sup>Safe (a-b) or ethidium bromide (c-f) for UV visualization.

The aligned amino acid sequence of wt pCYP17A1-VH, pCYP17A1-LH, pCYP17A1-VL, pCYP17A1-LL and pCYP17A1-LA are shown in Addendum B with amino acid residue differences indicated at positions 40, 105 and 407.

### 3.5.2 Analysis of prog conversion by wt pCYP17A1-VH

Wt pCYP17A1-VH was expressed in COS-1 cells and the catalytic activity of the recombinant enzyme determined by assaying the metabolism of 1 μM prog. Steroid metabolites were analysed by HPLC and a typical chromatogram obtained from HPLC analysis is shown in Figure 3-8.

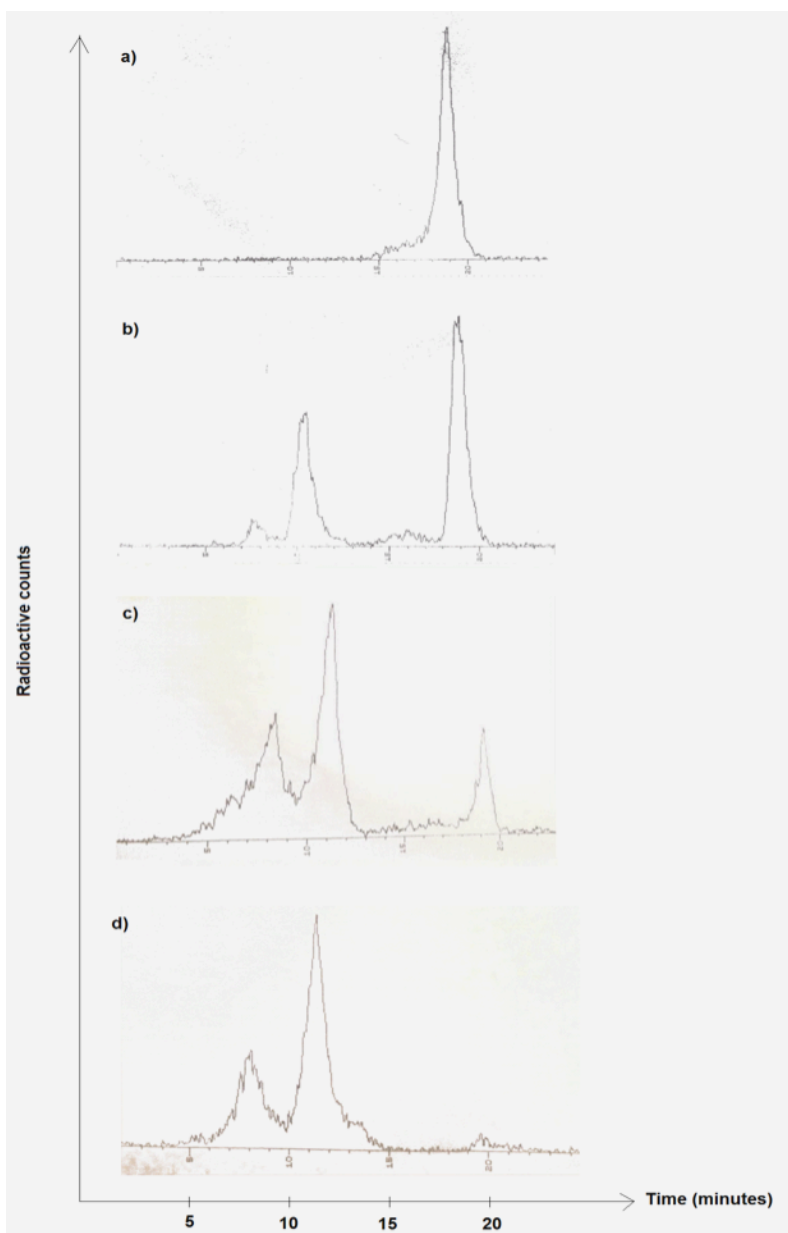


Figure 3-8: Typical HPLC chromatograms and elution profiles of A4 (8.93 min), 17OHprog (12.33 min) and prog (19.23 min) on a Novapak® C18 column, as described in materials and methods. Steroid profiles of prog metabolism by wt pCYP17A1-VH are shown at (a) T0; (b) T2; (c) T12 and (d) T24.

The retention times for the steroids separated by HPLC are depicted in Table 3-6.

Table 3-6: Retention time (min) of  $\Delta^4$  steroids separated by HPLC.

<b>Steroid</b>	<b>retention time (min)</b>
A4	8.93
17OHprog	12.33
prog	19.23



A time-course conversion assay (Figure 3-9) shows prog conversion over a 24 hr time period with samples taken at 2, 4, 8, 12 and 24 hr intervals.

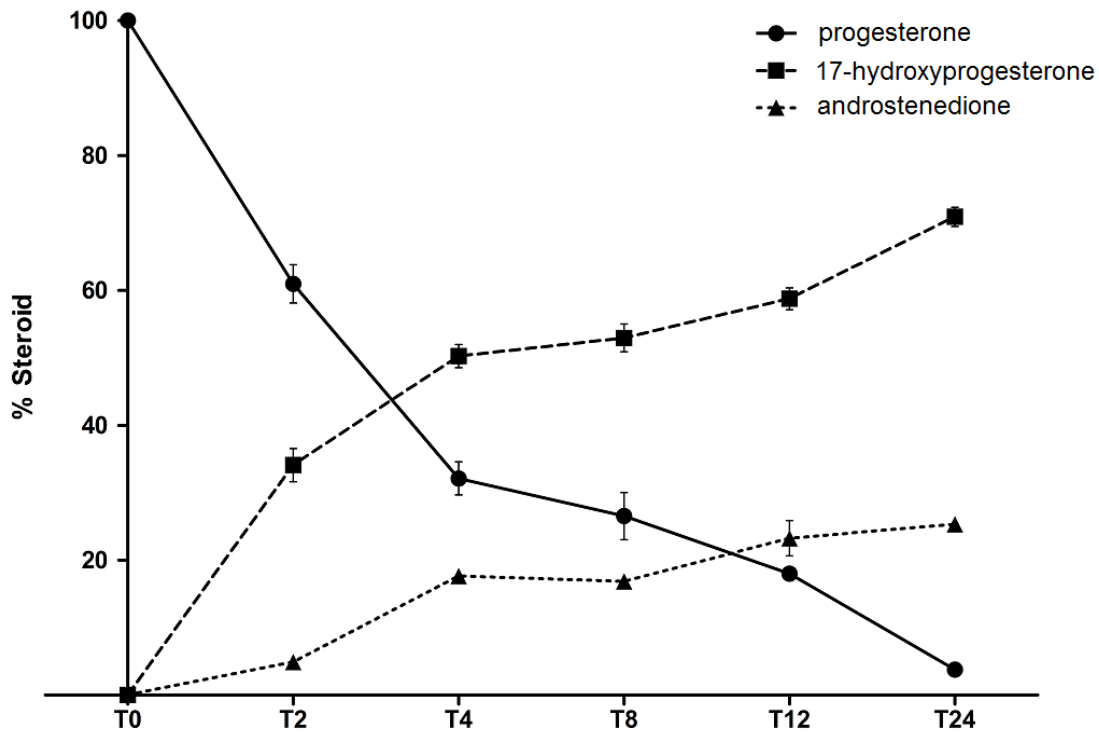


Figure 3-9: Metabolism of prog substrate, 1  $\mu$ M (2 000 000 cpm/ml  $^3$ H-prog), by wt pCYP17A1-VH. COS-1 cells were transfected with 0.5  $\mu$ g wt pCYP17A1-VH and 17OHprog and A4 production was analysed. Results are representative of three independent experiments (n = 9).

Within 3 hr, ~50% prog was metabolized to 17OHprog (~40%) and A4 (~10%). After 24 hr, negligible levels prog (~3.8%) remained with wt pCYP17A1-VH catalysing the production of ~70.9% 17OHprog and ~25.3% A4. No substrate conversion was detected in the assay in which COS-1 cells were transfected with pCI-neo containing no insert cDNA. Prog conversion by angora CYP17A1 was 50% at 1.5 hr with no prog remaining after 24 hr. Table 3-7 shows the percentage steroids present at each time interval.

Table 3-7: The percentage steroids present at (a) various hourly time (T) intervals during 1  $\mu$ M prog time-course conversion assay by wt pCYP17A1-VH and (b) 24 hr after 1  $\mu$ M prog conversion assay by angora CYP17A1.

DNA	Intervals (h)	prog %	17OHprog %	A4 %	17OHprog:17OHprog+A4
(a) wt pCYP17A1-VH	T0	100	0.00	0.00	0.00
	T2	61.0	34.1	4.90	0.87
	T4	32.1	50.2	17.6	0.74
	T8	26.5	52.9	16.8	0.76
	T12	18.0	58.8	23.3	0.72
	T24	3.80	70.9	25.3	0.74
(b) angora CYP17A1	T24	0.00	90.1	9.90	0.91

### 3.5.3 Analysis of prog conversion by wt pCYP17A1-VH with amino acid substitutions Val40Leu, His407Leu and Leu105Ala

Mutant constructs were expressed in COS-1 cells and prog conversion was assayed after 24 hr. UPLC-MS analyses detected 17OHprog, 16OHprog and A4. Figure 3-10 shows typical chromatograms obtained from UPLC-MS analysis, which elutes steroids according to the specified time schedule, as listed in Table 3-8.

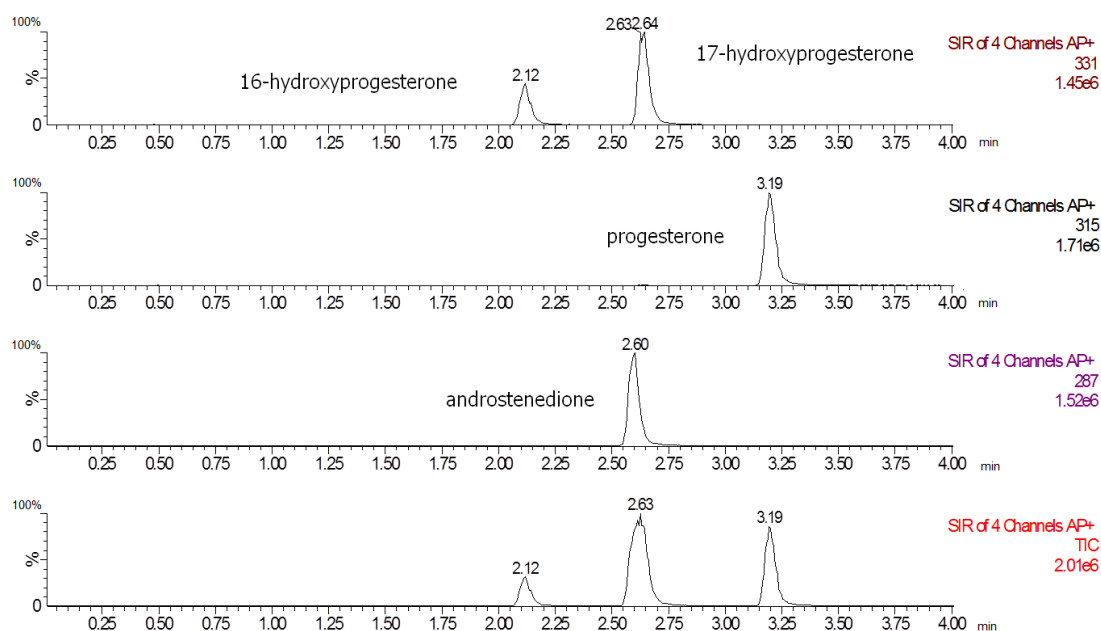


Figure 3-10: Selected ion chromatograms of  $\Delta 4$  steroid standards separated, according to retention times listed in Table 3-8, on a Waters UPLC BEH C18 column (2.1 mm x 100 mm, 1.7  $\mu$ m) at 50°C, as described in Materials and methods. Total ion current (TIC) of  $m/z$  200 to 400, for mixtures of standards, is also shown.

Steroids separated by UPLC-MS have the specifications ( $m/z$ ) and retention times as shown in Table 3-8.

Table 3-8: Peak identification, extracted ions and retention times of  $\Delta 4$  steroids.

Steroid	Extracted ion ( $m/z$ )	Retention time (min)
16OHprog	331	2.12
17OHprog	331	2.63
A4	287	2.60
prog	315	3.19

After 24 hr, ~98% of the total prog substrate was converted by wt and mutant clones: wt pCYP17A1-VH, pCYP17A1-LH, pCYP17A1-VL and pCYP17A1-LL (Figure 3-11). However, using UPLC-MS to detect the steroid metabolites, 17-, 16OHprog and A4 percentages showed to differ significantly. Subsequent analyses showed that there were no significant differences in 16OHprog production. Differences in the catalytic activity were evident when comparing the 17OHprog and A4, especially in the conversion by pCYP17A1-VL.

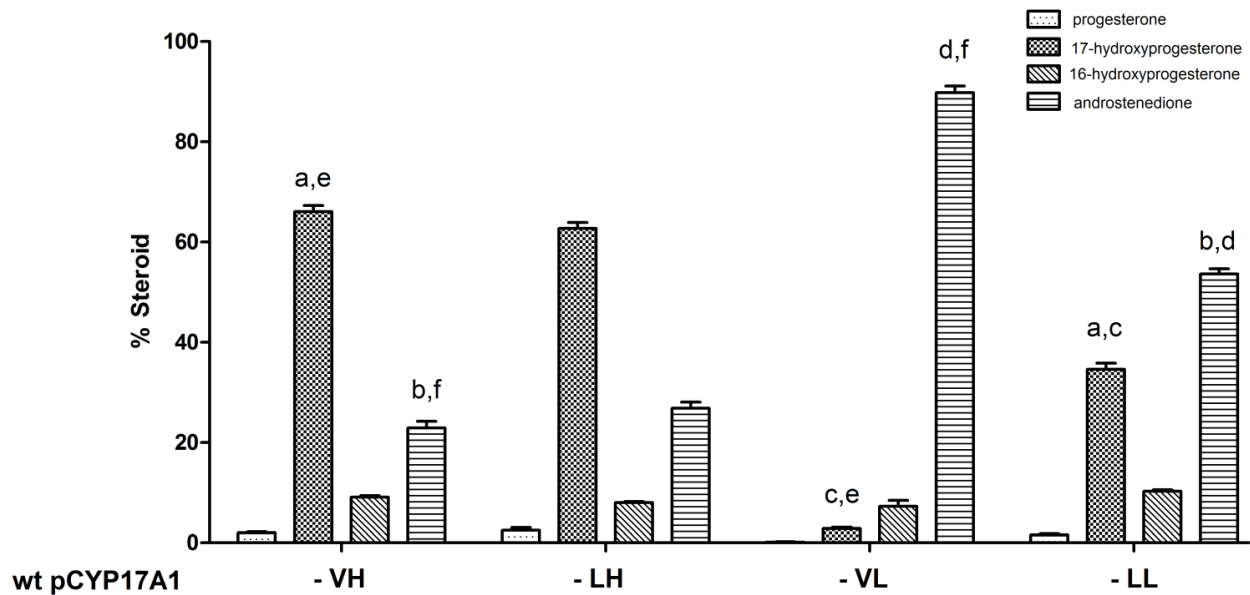


Figure 3-11: Steroid profiles of 1  $\mu$ M prog conversion after 24 hr by wt pCYP17A1-VH and mutants pCYP17A1-LH, -VL and -LL expressed in COS-1 cells. Results are representative of three independent experiments ( $n = 9$ ). Individual steroids were compared, represented by matching alphabetical *letters*, by a one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean  $\pm$ SEM. Comparisons of 17OHprog (a,c,e) percentages between wt and -LL (a); -VL and -LL (c); and between wt and -VL (e); as well as comparisons of A4 (b,d,f) percentages between wt and -LL (b); -VL and -LL (d); and between wt and -VL (f), were significantly ( $p < 0.0001$ ) different.

The conversion of prog by wt pCYP17A1-VH and pCYP17A1-LH were similar, producing metabolite levels not significantly different from each other (Table 3-9). However, conversion by pCYP17A1-VL showed a steroid profile significantly ( $p < 0.0001$ ) different to that of wt, pCYP17A1-LH and pCYP17A1-LL. In the conversion by pCYP17A1-VL, the levels of A4 produced were 3.9-fold more than that produced by wt pCYP17A1-VH, with a concomitant decrease of 23.6-fold in the levels of 17OHProg. Interestingly, a 2.3-fold increase in A4 was detected in the conversion by pCYP17A1-LL compared to wt.

Table 3-9: Percentage steroids present after 24 hr of 1  $\mu$ M prog substrate conversion by (a) wt pCYP17A1-VH, (b) wt mutants and (c) angora CYP17A1.

DNA	Time (h)	prog %	17OHprog %	16OHprog %	A4 %	17OHprog:17OHprog+A4
(a) wt pCYP17A1-VH	T24	1.90	66.1	9.10	22.9	0.74
(b) pCYP17A1-LH	T24	2.50	62.7	8.00	26.8	0.70
pCYP17A1-VL	T24	0.10	2.80	7.30	89.8	0.03
pCYP17A1-LL	T24	1.60	34.6	10.2	53.6	0.39
pCYP17A1-LA	T24	71.7	15.3	10.3	2.70	0.85
(c) angora CYP17A1	T24	0.0	86.4	2.96	10.6	0.89

pCYP17A1-LA decreased the overall catalytic activity of expressed enzyme when compared to wt, with 71.7% prog substrate remaining after 24 hr, (Figure 3-12), however, it would appear that the 16 $\alpha$ -hydroxylase activity was not affected by the mutation to the extent that the 17 $\alpha$ -hydroxylase was affected.

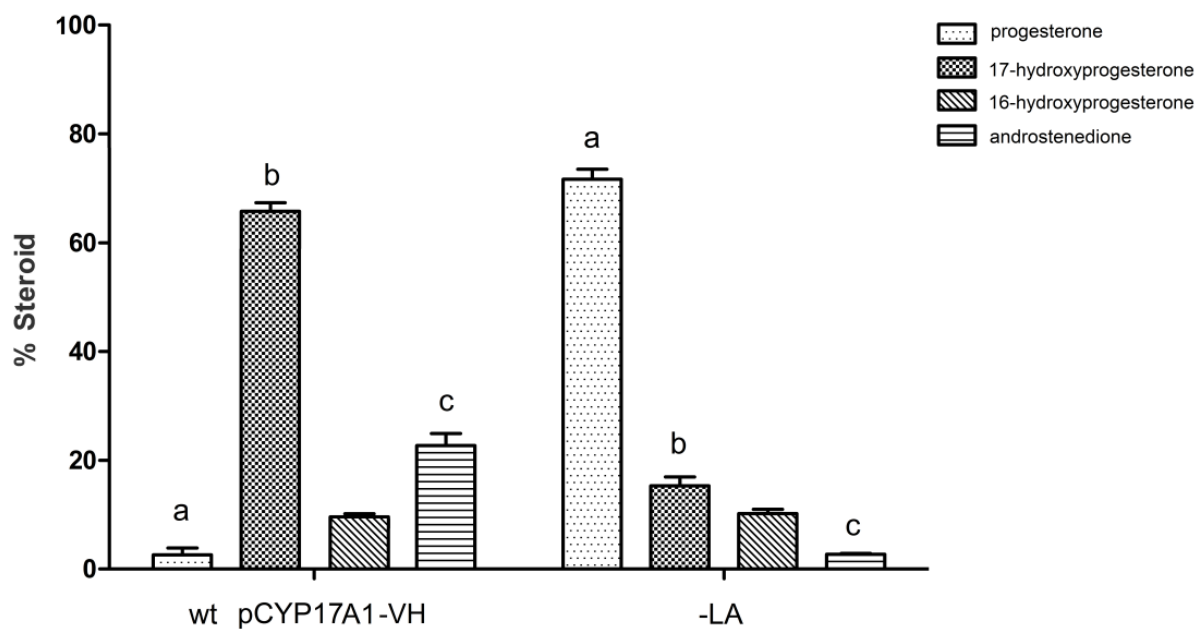


Figure 3-12: Prog conversion catalysed by wt pCYP17A1-VH and -LA (wt with L105A substitution). COS-1 cells were transfected with 0.5  $\mu$ g DNA and given 1  $\mu$ M prog substrate medium. Assays were taken after 24 hr. Steroids were separated by UPLC-MS, with a method separating prog, 17-, 16OHprog and A4. Results are representative of three independent experiments ( $n = 9$ ). Individual steroids were compared by a one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean  $\pm$ SEM. Comparisons between percentage prog (a), 17OHprog (b) and A4 (c) are significantly different ( $p < 0.0001$ ) from each other.

No substrate conversion was detected in the assay in which COS-1 cells were transfected with pCI-neo containing no insert cDNA. Prog was converted to steroid products by angora CYP17A1, with no prog remaining after 24 hr.

### 3.6 Discussion

In this study, three amino acid mutations were investigated, that at positions 40, 407 and 105. Sequence alignment of the deduced amino acid residues of wt pCYP17A1 differed from the published sequences for porcine adrenal and testes CYP17A1 proteins (see Addendum B). The amino acid sequences are identical, except for testes pCYP17A1 having a Leu407 residue and the adrenal protein having a His407 residue, while wt pCYP17A1-VH differs from these two sequences with residues Val40 and His407. Porcine wt CYP17A1 cDNA in the pCMV5 expression vector (wt pCYP17A1-VH) was used as template to construct four mutant pCYP17A1 constructs using site-directed mutagenesis and the plasmids were subsequently expressed in COS-1 cells. The catalytic activities, 17 $\alpha$ -hydroxylase/17,20-lyase and 16 $\alpha$ -hydroxylase activities were assessed by assaying the conversion of prog and analysing end-product formation.

In this study, mutants pCYP17A1-LL (testicular) and pCYP17A1-LH (adrenal) both have a Leu residue at position 40 and produce 17OHprog:A4 at a ratio of 0.64 and 2.33, respectively, after 24 hr. These enzymes differ only at position 407, with the testicular protein having a Leu residue and the adrenal protein having a His residue. However, maintaining the Leu residue at position 407 and replacing the Leu residue at position 40 with a Val residue (mutant pCYP17A1-VL) resulted in 17OHprog:A4 at a ratio of 0.03 after 24 hr. It would therefore seem that not only does the Val residue at position 40 influence the lyase activity of wt pCYP17A1-VH but the loss of the charged residue at position 407 also plays a significant role in the production of A4. Redox binding of both CPR and CYB5 have been shown to be more important for the lyase reaction than for the hydroxylation reaction. Position 40 lies upstream to the A helix (A' helix) and position 407 lies as the start of the meander region, downstream to the K' helix and just upstream of the heme-binding domain. The A-helix and K'-helix, in the crystal structure protein model, lie in close proximity to each other. Noteworthy, perhaps, is that changes in side-chains, such as the loss of the bulky charged side-chain of a residue (at position 407) and the insertion of a branched side-chain (at position 40), may influence the conformation of the protein — favouring the positioning of 17OHprog for the lyase reaction and thus involved in substrate

binding; interfere with the transfer and propagation of electrons from redox partners to the CYP17A1 heme center (specifically Cys442 and other neighbouring charged residues) and/or affect positioning of the heme-binding domain for inadequate acceptance of electrons — as the meander region is suggested to be involved in appropriate positioning of this domain.

Theoretical modelling of wt and mutants will show where the residues at positions 40 and 407 are in relation to secondary structural features, which are involved in-, essential to- and/or contribute to the redox partner binding sites of CYP17A1. A preliminary model shows that the His407 residue of wt and mutant porcine CYP17A1, is in fact positioned close to Arg residues, suggesting a possible interference with the redox partner as these charged residues have been shown to be vital for redox binding (Shimizu *et al.*, 1991).

End-products, 17OHprog and A4, of complete prog metabolism by CYP17A1 were measured after 24 hr and it would appear that it is only the conversion of 17OHprog to A4 that is significantly influenced by these mutations as the conversion of prog to 17OHprog was not noticeably affected by the different residues, at positions 40 and 407, when compared to wt 17OHprog formation after the same time period. These mutagenesis data do not reflect the rate of prog conversion by porcine CYP17A1, as only end-product formation was analysed, without determining dual reaction rate constants for wt and mutants. Therefore, differences observed in end-product formation are attributed to site-directed changes in the protein sequence.

Angora goat, which prefers the  $\Delta 5$  steroid 17OHpreg as the substrate for the 17,20 lyase activity, expresses two CYP17A1 isoforms CYP17A1ACS+ and CYP17A1ACS- (Storbeck *et al.*, 2007; Storbeck *et al.*, 2008a), both with residues Leu40 and Gln407, with only the latter catalysing the production of negligible A4 from 17OHprog in the presence of CYB5. Interestingly, at position 41, CYP17A1ACS+ has a Leu residue and CYP17A1ACS- has a Pro residue. Mutating the Pro residue of CYP17A1ACS- to a Leu residue led to a loss of lyase activity towards 17OHprog in the presence of CYB5, while the Leu41Pro mutation of CYP17A1ACS+ reinstated this activity under the same conditions. Although the effect of the residues at position 41 on the lyase activity was more pronounced with 17OHpreg as substrate, the differences detected were, nevertheless, significant. It is apparent that, while residues at positions 40 and 41 influence the lyase activity, these may be responsible for conformational changes, in conjunction with other residues, affecting structure in other domains.

While porcine CYP17A1 catalysed the conversion of 50% prog at ~3 hr, the conversion by the angora CYP17A1 isoforms are similar. In addition, the levels of 16OHprog catalysed by wt pCYP17A1-VH were the same after 24 hr, 7.3–10.2%, in all the assays conducted with the different constructs. This activity was further investigated by mutating the residue at position 105 in wt pCYP17A1, namely residue Leu105, to the residue, which is shown to be responsible for this activity in human CYP17A1, the residue Ala (Swart *et al.*, 1993; Swart *et al.*, 2010). The Leu105Ala substitution, however, generated a less active clone of wt pCYP17A1, with the 17 $\alpha$ -hydroxylase activity of the enzyme for prog significantly reduced. With more than 70% prog remaining after 24 hr, the 16 $\alpha$ -hydroxylase activity seemed unaffected. However, further analysis of the data shows that the ratio of 16OHprog:17OHP4+A4 does significantly change from 0.10 to 0.57 after the Leu105Ala mutation, which strongly suggests that the mutation itself is responsible for the increased change in this ratio and, with 16OHprog levels remaining unaffected, the mutation thus affected only the 17 $\alpha$ -hydroxylase activity.

The aim of chapter 3 was to assess the influences of three amino acid substitutions on the activity of porcine wt CYP17A1 towards prog. After complete prog metabolism at 24 hr, end-product comparisons indicated that the conversion of 17OHprog to A4 was significantly influenced by residues at positions 40 and 407, while the conversion of prog to 17OHprog was not affected. As CYB5 is known to influence the lyase activity, porcine CYB5 was subsequently cloned and its influence on the catalytic activity of wt pCYP17A1-VH was investigated and is described in the next chapter.



## CHAPTER 4

### THE INFLUENCE OF SPECIES-SPECIFIC CYB5 ON THE 17,20 LYASE ACTIVITY OF PORCINE CYP17A1

#### 4.1 Introduction

Membrane-bound CYB5 is a highly conserved heme protein consisting of 134 amino acids and has a predicted molecular weight of ~16 kDa. This small, electron transfer protein is widely expressed. Adrenal and testes expressed CYB5 serves as a modifier of the steroidogenic cytochrome P450, CYP17A1 (Schenkman and Janssom, 2003). Numerous structure based studies have provided a plethora of valuable information describing the precise mechanism by which CYB5 and CPR interact with and facilitate CYP17A1 activity (Omata *et al.*, 1994a, Omata *et al.*, 1994b; Dürr *et al.*, 2007).

CYP17A1 is capable of catalysing the transformation of four different C21 steroid substrates at a common active site (Miller and Auchus, 2011). After the first round of oxidation, either hydroxylated products are shunted towards other enzymes for the formation of corticoids or they are exposed to the second catalytic reaction of CYP17A1, which represents the first dedicated step in androgen synthesis. The specificity of this enzyme towards its substrates differs in various species and tissues with most variation being attributed to 16 $\alpha$ -hydroxylase and 17,20-lyase activity (Swart *et al.*, 1993; Swart *et al.*, 2010; Miller and Auchus, 2011). The chemical mechanisms of these reactions have evoked immense interest, particularly with the chemistry regarding the carbon-carbon cleavage and the structure-function relationship of all the components enabling, facilitating or hampering this action (Mathieu *et al.*, 2003; Gregory *et al.*, 2013).

Essentially, CYP17A1 activity is dependent on the initial reduction of the heme group by the acquisition of electrons from the readily available redox partner CPR (Shimada *et al.*, 2005). The involvement of CYB5 is dependent on species, tissue-specific expression and the substrate involved. CYB5 has been shown to have considerable influence on the  $\Delta$ 5- and  $\Delta$ 4 pathways, stimulating the 17,20-lyase activity of CYP17A1 as well as the activity of 3 $\beta$ HSD, while not influencing other steroidogenic enzymes (Akhtar *et al.*, 2005; Goosen *et al.*, 2011). For the insertion of molecular oxygen into a steroid substrate, CPR provides both the required electrons. Hydroxylation occurs primarily at C17 and in certain species, C16, due to the precise molecular alignment of CYP17A1 with the

substrate, which is achieved by the orientation of the CPR-P450 in complex with each other (Akhtar *et al.*, 2005). In species susceptible to CYB5 stimulation, CYB5 facilitates the transfer of the second electron and the conformational change that is induced in the active site of CYP17A1, when CYB5 interacts with this complex, is such that the oxidative attack by the iron-oxygen intermediate of CYP17A1 is redirected to C20 resulting in the scission of the C17-20 bond. At low concentrations, CYB5 may enhance the rate of enzyme catalysis up to 100-fold; however at high concentrations it inhibits catalysis by competing with CPR for a binding site on the P450 enzyme, thus preventing the transfer of the first electron and consequent reduction of heme (Im and Waskell, 2011).

The molecular dynamics of hemeproteins in complex with substrates have been well documented, identifying structural motifs and amino acid residues crucial to proper complex formation and optimal enzyme functioning (Auchus and Miller, 1999; Ahuja *et al.*, 2013).

#### 4.2 Structural domains of CYB5 determined by NMR spectroscopy

To date, several NMR spectroscopy- and X-Ray crystallography based studies have elucidated the 3D tertiary and crystal structures, respectively, of various species truncated variants of microsomal membrane CYB5, including bovine CYB5 (pdb-id's 1HKO, Muskett *et al.*, 1996 and 1CYO, Durley and Mathews, 1996); rat CYB5 (1AW3, Arnesano *et al.*, 1998), rabbit CYB5 (1DO9, Banci *et al.*, 2000) and human CYB5 (2I96, Nunez-Quintana *et al.*, 2010). Recently, a 3D model of membrane-bound mammalian CYB5, as well as its quaternary structure in complex with a P450, has been developed. Ahuja *et al.* (2013) reported the first structure of full-length rabbit ferric microsomal CYB5 (pdb-id 2M33), incorporated in two different membrane types (detergent micelles and lipid bicelles) (Ahuja *et al.*, 2013).

The structure of the soluble heme domain of CYB5 contains five  $\alpha$ -helices, five  $\beta$ -strands and one  $3_{10}$  helix (Pro86-Arg89), which marks the end of this globular domain (Ahuja *et al.*, 2013). Residues Lys10-Tyr12 form the first  $\beta$ -sheet ( $\beta$ 1) and residues Leu14-His20 form the first  $\alpha$ -helix ( $\alpha$ 1). The heme binding section consists of two helices in the lower cleft, shown as  $\alpha$ 4 (Thr60-Val66) and  $\alpha$ 5 (Thr70-Phe79); two helices in the upper cleft, shown as  $\alpha$ 2 (Lys39-Glu43) and  $\alpha$ 3 (Glu49-Gln54) and three  $\beta$ -sheets at the bottom of the heme pocket, shown as  $\beta$ 3 (Lys33-Asp36),  $\beta$ 2 (Trp27-Leu30) and  $\beta$ 4 (Gly56-Asp58). Figure 4-1 shows the sequence and annotated secondary structures of full-length rabbit CYB5 aligned with sequences and NMR structures of truncated CYB5 from various other species.

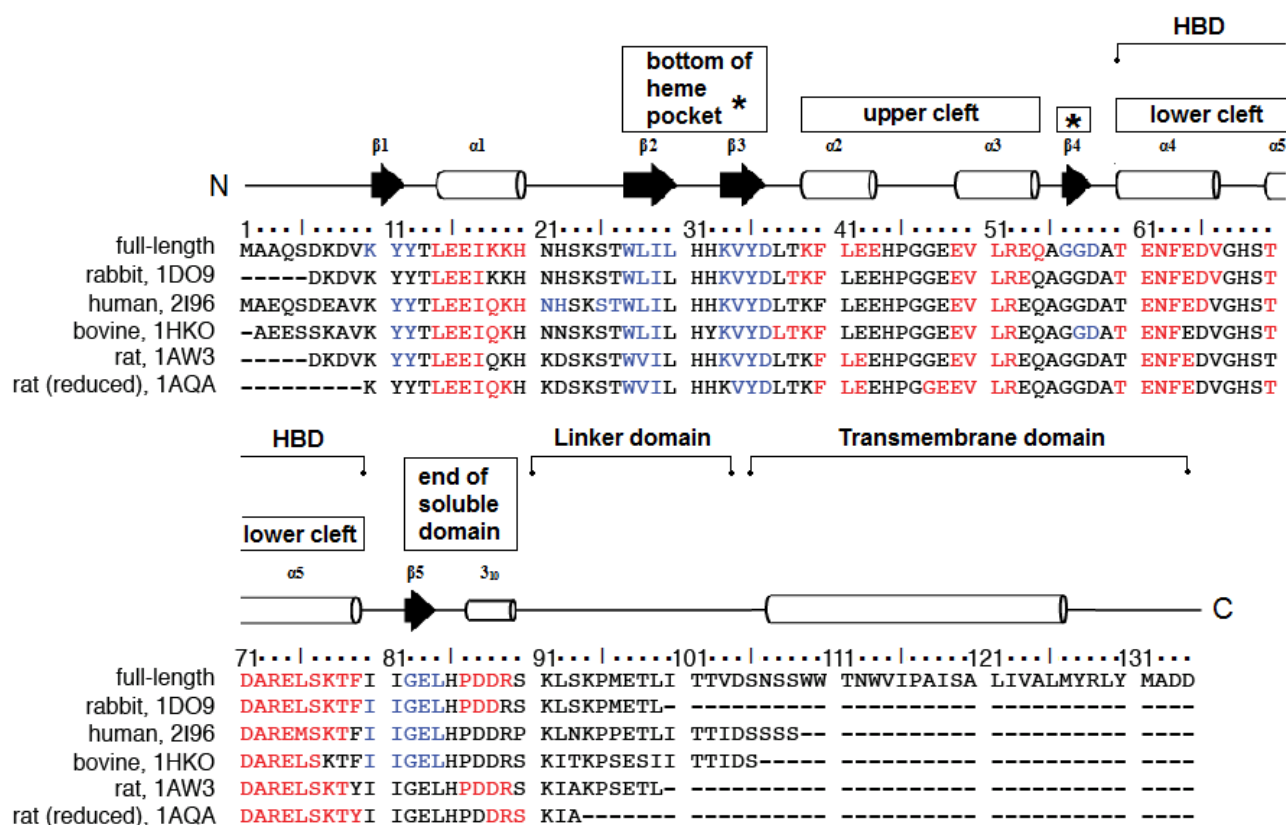


Figure 4-1: Sequence and secondary structure comparison of full-length rabbit CYB5 with truncated variants from various species. The following species amino acid sequences are presented: rabbit full-length (pdb-id [2M33](#)) and truncated rabbit (1DO9), human (2I96), bovine (1HKO), rat (1AW3) and reduced rat (1AQA). Amino acid sequences that are involved in the secondary structural elements  $\alpha$ -helices (red) and  $\beta$ -sheets (blue) are highlighted. Heme association occurs above  $\beta$ -sheets 2-4, and between  $\alpha$ -helices 4 and 5 in the heme-binding domain (HBD). Conserved residues His44 and His68 coordinate heme positioning. One side of the heme is exposed through the opening of the structural cleft between  $\alpha$ -helices 2 and 3. Domains and other structural features are also shown (reproduced from Ahuja *et al.*, 2013, and supplementary data).

The overall structure of the heme domain of full-length ferric CYB5, with residues His44 and His68 shown to coordinate with the heme group, was found to be similar to the previously determined NMR structure of the heme domain of truncated ferric rabbit CYB5 (Banci *et al.*, 2000; Ahuja *et al.*, 2013). These two heme-orientating residues appear to be conserved for known sequences of species CYB5 (see Addendum C). CYB5 exists as two isomers (major and minor), which differ by a 180° rotation of the heme axis that cuts through the *meso*-carbon atoms  $\alpha$  and  $\gamma$ . The major/minor isomer ratio of full-length rabbit CYB5 was determined to be 6.6:1 (Ahuja *et al.*, 2013), which is similar to the 5:1 ratio determined for truncated rabbit CYB5 (Banci *et al.*, 2000) and nearly identical to the 6.5:1 isomer ratio for truncated bovine CYB5 (Zhang *et al.*, 2004). The isomer ratio depends on the CYB5 species and it has been reported to be as high as 1.5:1 for rat CYB5, despite sequence similarity between rabbit and rat CYB5 (Lee *et al.*, 1990).

The linker domain (Ser90-Asp104) connects the cytosolic heme domain to the transmembrane domain (Ser105-Asp133) (Ahuja *et al.*, 2013). The full-length CYB5 linker region appears unstructured; a random coil lacking any distinct secondary features. It has been shown that in this region, at least 6-8 amino acids are necessary to enable formation of a functional complex between CYB5 and its full-length redox partner cytochrome P450 (Clark *et al.*, 2004). The transmembrane domain is conserved among vertebrates with sequence similarity of 78-96% and is essential for complex formation with redox partners (Vergeres and Waskell, 1992). With respect to perpendicular insertion in the bilayer, a 15° average tilt angle has been calculated for the transmembrane  $\alpha$ -helical anchor (Asn106-Met126) Ahuja *et al.*, 2013).

Figure 4-2 shows a ribbon diagram of the structural model of the 134 amino acid rabbit CYB5.

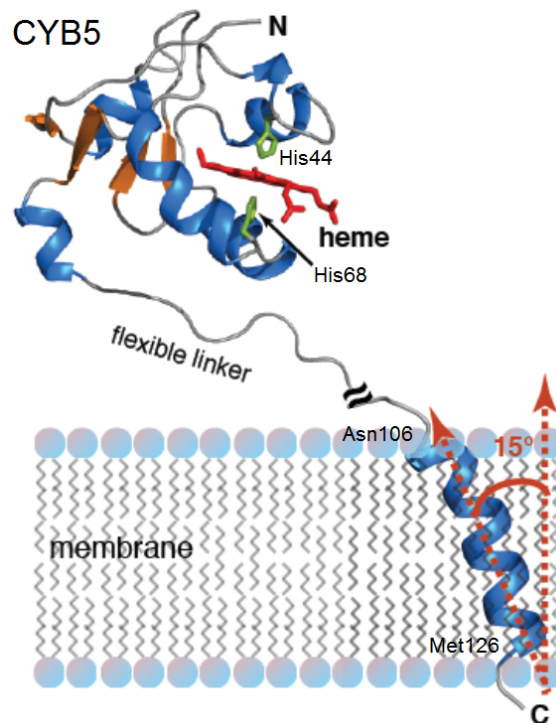


Figure 4-2: Ribbon diagram of the 3D NMR structure of full-length rabbit CYB5 with  $\alpha$ -helices (*blue*) and  $\beta$ -sheets (*orange*). The N-terminal soluble heme domain (residues 1-Arg89); flexible linker and angled C-terminal transmembrane domain, with  $\alpha$ -helix anchor (Asn106-Met126), are shown. Residues (*green*) His44 and His68 coordinate heme group (*red*) positioning (reproduced from Ahuja *et al.*, 2013).

### 4.3 Redox partner binding site and CYP17A1 activity

Human CYP17A1 favours  $\Delta 5$ - and  $\Delta 4$  steroid  $17\alpha$ -hydroxylation that occurs at the most reactive C-H bond in the steroid substrate and is relatively insensitive to changes in the P450-CPR conformation. The more intricate reaction of C-C cleavage, that few species catalyse, can only occur in a narrow range of P450-CPR geometries. Allosteric action by CYB5 optimizes the topology of this complex, thus markedly stimulating scission of the bond.

The redox-partner binding lies on the opposite side of the heme group away from the substrate-binding site (Ravichandran *et al.*, 1993). There are many basic residues situated in this region (mostly Arg residues in CYP17A1), which yield a surface charge distribution that is predominantly positive. The surfaces of the FMN domain of CPR and CYB5, which interact with the P450, contain a collection of acidic residues. CYB5 and CPR thus compete for an overlapping binding site on the P450 molecule, rather than having separate functional binding sites (Bridges *et al.*, 1998). The protein surfaces interacting remain separated by several angstroms and so the interactions between these oppositely charged residue clusters are weak and indirect (Ahuja *et al.*, 2013).

CYP17A1 residues Lys89, Arg347 and Arg358 are highlighted specifically to be involved with the redox binding proteins (Auchus *et al.*, 2000). Neutralizing these Arg residues in the CYP17A1 redox binding site, individually result in selective loss of  $17,20$ -lyase activity (Geller *et al.*, 1997). Although there is a subtle disturbance in the CYP17A1-CPR complex, electron transfer can still take place when these basic residues are neutralized (Auchus and Miller, 1999).

CYP17A1 mutation Pro342Thr, which is located upstream of Arg347 in the  $J'$ -helix, causes partial loss of both  $17\alpha$ -hydroxylase and  $17,20$ -lyase activities (Ahlgren *et al.*, 1992). The resulting Thr-Thr-Thr motif is suggested to extend and misdirect the  $J'$ -helix, ultimately creating a significant change in the structure of the redox-binding site (Auchus and Miller, 1999).

CYP17A1 Phe417 lies adjacent to, and not within, the redox partner binding site (Miller and Auchus, 1999). However, mutations at this position (Phe417Cys) also selectively disrupt  $17,20$ -lyase activity (Biaison-Lauber *et al.*, 1997). The human CYP17A1 model (pdb-id [2C17](#)) shows that Phe417 lies C-terminal of the meander sequence (His407-Arg416), which can not be accessed by solvent and thus cannot form part of the redox-binding site. The authors suggested that although Phe417 is not a component of the redox-binding site, it does form an edge for the redox-binding site by contributing to

hydrophobic interactions that assists in stabilizing the “flap” (sequence adjacent to Arg358 and lacking basic residues) between the heme-binding domain and the meander (Auchus and Miller, 1999).

Human CYB5 with double mutation Glu48Gly and Glu49Gly, which introduces a very flexible sequence of four Gly residues, has been shown to be deficient in its ability to stimulate the activity of human CYP17A1 (Naffin-Olivos and Auchus, 2006). This observation was consistent with the presence of Glu48 and Glu49 in the interaction interface of cluster II and considerable line broadening NMR data was observed for CYB5 Glu48 and Glu49 upon complex formation with the CYP2B4 (Ahuja *et al.*, 2013).

Remarkable differences in CYP17A1 activities occur among species and conserved key residues have been identified to be involved in- and are essential to hemeprotein interactions and activity. Current experimental procedures, the extent of data obtained and the accuracy of tested prediction models of hemeproteins, all provide a strong foundation for attributing structure/function similarities to different species of the same protein.

There is modest amino acid sequence identity occurring between the ~500 amino acids of various species CYP17A1s and a greater identity occurring between the 134 amino acids of various species CYB5s. Therefore, the aim of this chapter will be to observe whether there is a specific relationship or interaction between same species CYP17A1 and CYB5 in conferring maximal activity by that species CYP17A1 protein. This will be achieved by investigating whether or not wt pCYP17A1-VH activity is amplified in a similar manner when co-expressed with alternative species CYB5 cDNA.

Porcine CYB5 cDNA will be cloned from porcine liver homogenates by reverse transcribing cDNA from mRNA. Co-expression assays of wt pCYP17A1-VH with porcine CYB5 (pCYB5), human CYB5 (hCYB5), angora CYB5 (aCYB5) and rat CYB5 (rCYB5) will be conducted in COS-1 cells with prog metabolism assayed. Testicular pCYP17A1 (-LL) will also be assayed with pCYB5 and end-products will be compared to those assayed with adrenal pCYP17A1 (-VH).

Porcine CYB5 shares 86.5% amino acid sequence identity with human CYB5 (18 amino acid differences), 90.2% with angora CYB5 (13 amino acid differences) and 91.7% with rat CYB5 (11 amino acid differences). Figure 4-3 shows the full-length amino acid sequence alignments of porcine-, human-, angora- and rat- CYB5 annotated with inferred secondary structural features.

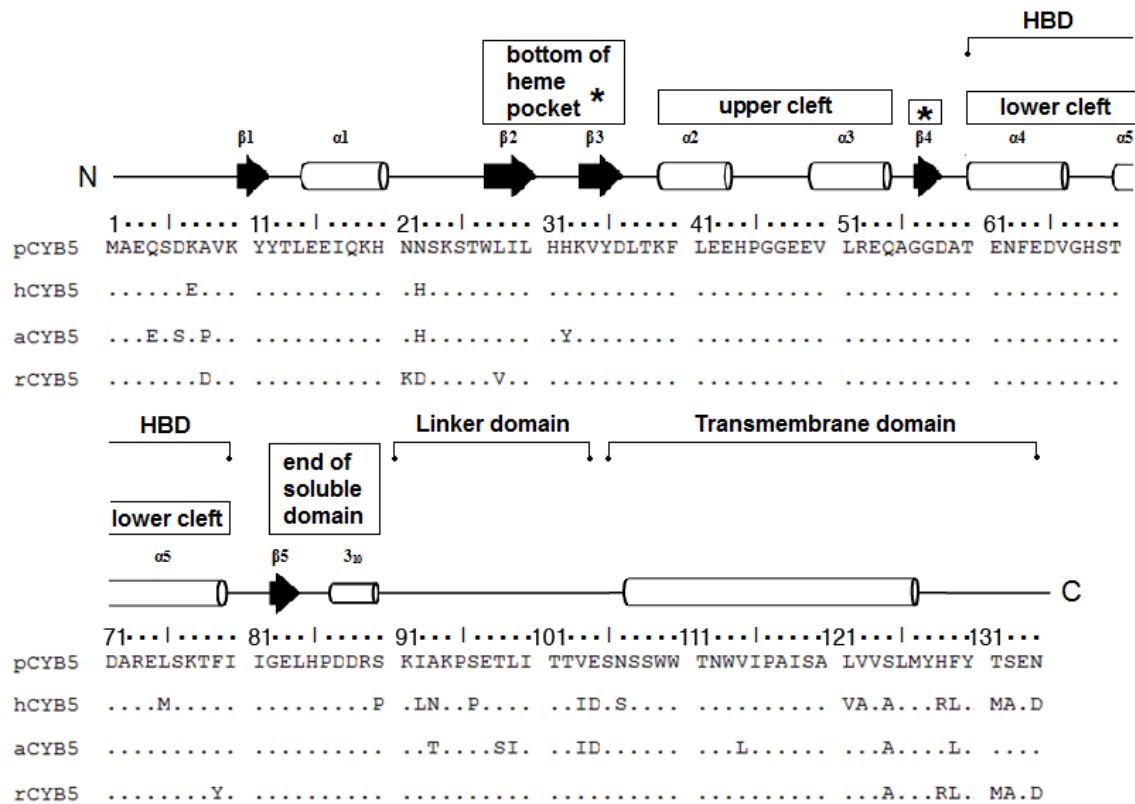


Figure 4-3: Amino acid sequence alignments of full-length species CYB5 used in the co-expression assays with wt porcine CYP17A1. The GenBank accession numbers for species CYB5 are: porcine CYB5 (pCYB5) [P00172.3](#); human CYB5 (hCYB5) [P00167.2](#); angora CYB5 (aCYB5) [ABQ12619.1](#) and rat CYB5 (rCYB5) [P00173.2](#). hCYB5, aCYB5 and rCYB5 are aligned with regards to the pCYB5 sequence. Secondary structure elements are shown by barrels ( $\alpha$ -helices) and arrows ( $\beta$ -sheets) (inferences from Ahuja *et al.*, 2013).

## 4.4 Materials and methods

### 4.4.1 Plasmids

Human pCMW\_CYB5 (hCYB5), angora pCMW\_CYB5 (aCYB5) and rat pCW\_CYB5 (rCYB5) and control plasmids, pCI-neo and angora CYP17A1 (aCYP17A1), were available for use in the laboratory. Their sequences correspond to their GenBank counterparts (protein identification numbers): aCYP17A1 ([ABQ12616.1](#)) and hCYB5 ([P00167.2](#)), aCYB5 ([ABQ12619.1](#)), rCYB5 ([P00173.2](#)) are aligned in Figure 4-3.



#### 4.4.2 Reagents

<sup>3</sup>H-Prog was purchased from PerkinElmer Life Sciences (Boston, MA, USA). Prog, 17OHprog, 16OHprog, A4 and DMEM were purchased from Sigma Chemical Company (St. Louis, MO, USA). COS-1 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). Penicillin-streptomycin and trypsin-EDTA were purchased from Gibco-BRL (Gaithersburg, MD, USA). Fetal calf serum and bacterial culture media were purchased from Highveld Biological (Lyndhurst, RSA). GeneJuice transfection reagent was purchased from Novagen (Darmstadt, Germany). Wizard® Plus SV Minipreps DNA Purification system and DNA markers ( $\lambda$ DNA/*EcoRI*+*HindIII* and 1 kb marker) were purchased from Promega Biotech (Madison, WI, USA). TRI-Reagent® Kit and nuclease-free water were purchased from Ambion, an Applied Biosystems Business (Austin, Texas, USA). RNase-free plasticware, oligoDT (deoxythymidine), ribonucleotide phosphates and primers were purchased from Whitehead Scientific (Pty) Ltd (Bellville, RSA). 1<sup>st</sup> Strand cDNA Synthesis kit for RT-PCR (AMV), PWO DNA Polymerase High Fidelity, restriction endonuclease *AcsI* and DNA marker, *marker VI* (digested pBR328 DNA/*BglI* and pBR328 DNA/*HinfI*) were purchased from Roche-Applied Sciences (Mannheim, Germany). pcDNA™ Gateway® Directional TOPO® Expression kits and One shot® TOP10 *E. coli* cells were purchased from Invitrogen (Carlsbad, USA). Nucleobond® AX plasmid preparation kits were purchased from Machery-Nagel (Duran, Germany). PCR machines, electrophoresis apparatus and spectrophotometers are of the highest laboratory standards. All other chemicals were of the highest analytical grade and purchased from scientific supply houses.

#### 4.4.3 RNA isolation and cDNA cloning

Porcine livers were obtained at Winelands Pork, Stikland, abbatoire facility (Mr. Fredi Williams). Multiple 5 x 3 cm Landros porcine liver sections were collected 20 min after slaughtering, using the necessary sterile precautions. Sections were immediately contained in sterile RNase-free 50 ml falcon tubes and stored in liquid nitrogen.

Extraction and purification of porcine liver RNA was conducted at 4°C, with the necessary precautions taken in handling RNA and avoiding RNase contamination (Ausubel *et al.*, 1991). Upon thawing, liver sections, ~50 mg, were sliced and placed into 1.5 ml RNase-free centrifuge tubes containing Tri-Reagent®, 500  $\mu$ l. Samples were homogenized at 4°C using a glass homogenizer after which the



homogenate was transferred into RNAase-free 1.5 ml centrifuge tubes containing equal volume Tri-Reagent®. Total RNA isolation was performed at room temperature using the TRI-Reagent® total RNA isolation kit, according to manufactures instructions and was quantified by absorbance at 260/280 nm.

Reverse transcriptase PCR was performed with a RNA reaction mixture containing RNA, 2 µg, oligodT's (0.5 µg/µl), 2 µl, and nuclease-free water, in a total volume of 11 µl. Samples were incubated on a heating-block at 70°C for 5 min to denature RNA secondary structures. Thereafter, samples were placed on ice for 5 min to allow annealing of the dT primer to mRNA. The components of the 1<sup>st</sup> strand cDNA synthesis RT-PCR: AMV-RT buffer (5X), 1X, 5 µl, RNase inhibitor (50 U/µl), 50 U, 1 µl, AMV reverse transcriptase, 3 µl, and nuclease-free water were added together in a total reaction volume of 14 µl. The reverse transcriptase reaction mixture was added to the RNA reaction mixture and was placed on the heating-block at 42 °C for 1 hr.

The cDNA obtained was used as template DNA in the PCR amplification to generate double stranded porcine DNA using primers listed in Table 4-1. Proof reading polymerase (PWO DNA Polymerase High Fidelity) was used, according to manufacturers' instructions in the amplification reaction to ensure that incorrect nucleotides were not incorporated into the amplified DNA.

Table 4-1: Primer sequences used in the PCR amplification and direct DNA sequencing of pCYB5. First left and right primer pair (LP1 and LP2) are complementary to the 5' and 3' ends of porcine CYB5 (GenBank protein identification number P00172.3), respectively. The CACC sequence (underlined) was incorporated in LP1, which was required for subsequent TOPO cloning.

Primer	Oligonucleotide sequence
LP1 (Sense)	5'- <u>CACCCTCGCTGAGTTAAGAAATG</u> -3'
RP1 (Antisense)	5'-CTGCTTTGGTCCAGGGAG-3'

The PCR components were combined to a total reaction mixture of 100 µl with nuclease-free water: MgSO<sub>4</sub>-PCR buffer (10X), 1X, 10 µl; dNTPs (10 mM), 400 µM, 4 µl; each primer (20 µM), 300 nM, 1.5 µl; PWO (5 U/µl), 2.5 U, 0.5 µl, and RT-PCR product, 3 µl. PCR amplification was performed under the following conditions: denaturation at 94°C for 2 min; annealing at 94°C for 30 sec, 55°C for 30 sec, 70°C for 1 min (35 cycles) and the extension stage at 70°C for 10 min.

A pcDNA<sup>TM</sup> Gateway® Directional TOPO® expression kit was used, according to manufacturers' instructions. PCR product, porcine CYB5 cDNA, 4 µl, was ligated into the mammalian expression vector TOPO® (pcDNA3.2/V5/GW/D-TOPO®) (15-20 ng/µl), 1 µl, in a salt solution (1.2 M NaCl;

0.06 M MgCl<sub>2</sub>), 1 µl. The reaction was incubated at room temperature for 30 min and then placed on ice. One shot® TOP10 *E. coli* cells were transformed with the addition of PCR amplification mixture, 2 µl, and incubated on ice for 30 min. Thereafter, the reaction was heat shocked in a water bath at 42°C for 30 sec and placed back on ice for 1 min. Room temperature SOC medium, 250 µl, was added after which the cells were incubated at 125 rpm at 37°C for 1 hr. Cells were plated on LB medium-agar containing ampicillin, 100 µg/ml, and grown overnight at 37°C with petri dishes placed upside-down.

LB medium, 5 ml, containing ampicillin, 100 µg/ml, was inoculated with a single colony of transformed TOP10 *E. coli* cells and incubated overnight at 37°C at 225 rpm. Plasmid DNA was isolated using the Wizard® Plus SV minipreps DNA purification system, according to manufacturers' instructions. Plasmid DNA, 10 µl, was subjected to a restriction digest for 1.5 hr in a 50°C warm bath, with 10 U restriction enzyme *AcsI* (10 U/µl), according to manufactures instructions. The products were separated by 1% agarose gel electrophoresis at 55 V with a DNA marker, *1 kb marker* (Promega) and stained with ethidium bromide for UV visualization. Positive clones were subsequently subjected to sequence analysis with universal primer T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and primers listed in Table 4-1. All DNA sequencing was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster city, California).

LB medium, 100 ml, containing ampicillin, 100 µg/ml, was inoculated with freezer stocks of pcDNA-CYB5, 100 µl, and incubated overnight at 37°C at 225 rpm. The plasmid DNA was isolated using the Nucleobond® AX100 DNA Isolation Kit, according to manufacturers' instructions (Machery-Nagal). After precipitation, the plasmid DNA pellet was redissolved in deionized water, 200 µl, and the plasmid yield was calculated by UV spectrophotometry, 260/280 nm.

#### **4.4.4 Enzymatic assays in non-steroidogenic mammalian COS-1 cells**

COS-1 cells were grown to confluence in 100 mm culture dishes at 37°C and 5% CO<sub>2</sub> in DMEM, 10 ml; supplemented with 0.9 g/L glucose, 0.12% NaHCO<sub>3</sub>, 10% fetal calf serum and 1% penicillin-streptomycin (stock containing 10 000 U/ml penicillin and 10 000 µg/ml streptomycin). Twenty four hours prior to transfection, cells were plated into 12-well culture dishes with each well containing 1×10<sup>5</sup> cells (1 ml/well). For co-transfection experiments, cells were transfected with medium containing transfection reaction mix (1 ml/well), 50 µl, which contained total plasmid DNA of 0.5 µg (two different cDNAs of 0.25 µg each) and GeneJuice transfection reagent, 1.5 µl. Control transfection

reactions were performed using the mammalian expression vector pCI-neo (containing no gene insert) and a positive control plasmid, angora CYP17A1. The cells were incubated for 72 hr, after which enzyme activity was assayed by adding steroid substrate 1  $\mu$ M prog with or without  $^3$ H-prog (100 000 cpm/50  $\mu$ l) to fresh growth medium. Aliquots of 50  $\mu$ l or 500  $\mu$ l were removed at specific time intervals to assay substrate conversion and were analysed by HPLC or UPLC-MS, respectively. Deionised water, 450  $\mu$ l was added to the tritiated samples after which steroid metabolites were extracted in dichloromethane, 5 ml, for analyses by HPLC. Non-tritiated samples were extracted in dichloromethane, 5 ml and steroid metabolites analysed by UPLC-MS. The dichloromethane phase was evaporated under a stream of nitrogen. Dried steroid residue was redissolved in methanol, 120  $\mu$ l, prior to analysis. After the last time-point, cells were washed with and collected in phosphate buffer (0.1 M, pH 7.4) and sonicated prior to determining the protein content by the Pierce BCA method (Pierce Chemical) according to the manufacturers' instructions.

#### **4.4.6 Separation and quantification of steroid metabolites**

The chromatographic separation of steroids was performed on a Waters (Milford, MA, USA) HPLC coupled to a WISP™ automatic injector (Waters) and a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL). Prog, 17OHprog and A4 were separated on a Novapak® C18 column at a flow rate of 1 ml/min. The mobile phase consisted of solvent A (35% water and 65% methanol) and solvent B (100% methanol). The column was equilibrated for 10 min with solvent A, followed by a linear gradient from 100% solvent A to 100% solvent B in 5 min, after which an isocratic elution of solvent B ran for 2 min.

Steroid metabolites, prog, 17OHprog, 16OHprog and A4 were separated and quantified using UPLC-MS on a Waters UPLC BEH C18 (2.1 mm  $\times$  100 mm, 1.7  $\mu$ m) column (ACQUITY UPLC, Waters, Milford, USA). The mobile phases consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B). Steroids were eluted at a flow rate of 0.35 ml/min, starting with the mobile phase 85% solvent A and 15% solvent B for 3.5 min, followed by 20% solvent A and 80% solvent B for 2.1 min, followed by 100% solvent B for 1 min and finally 85% solvent A and 15% solvent B for 2 min. An API Quattro Micro tandem mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. An Ion Sabre probe (Waters, Milford, USA) was used for the APCI interface in positive mode. The corona pin was set to 7  $\mu$ A, the cone voltage to 30 V and APCI probe temperature was

100°C. All other settings were optimized to obtain the strongest signal possible. Data was collected with the MassLynx 4.0 software program.

## 4.5 Results

### 4.5.1 Cloning and analyses of pCYB5

Total RNA was isolated from porcine liver homogenates after which RNA was reverse transcribed using oligodT primers and porcine cDNA encoding CYB5 was amplified by RT-PCR. The RT-PCR product was subsequently cloned into the pcDNA3.2/V5/GW/D-TOPO<sup>®</sup> vector and transformed into *E. coli* cells. Isolated plasmid DNA of clones were subjected to digestion by restriction enzyme *AcsI*. The size of vector containing porcine CYB5 cDNA is shown in Table 4-2, as well as the sequence recognition sites for restriction enzyme *AcsI*.

Table 4-2: Recognition sites of endonuclease *AcsI* recognition sites in (a) expression vector, pcDNA3.2/V5/GW/D-TOPO<sup>®</sup>; (b) linear porcine CYB5 cDNA and (c) porcine CYB5 cDNA ligated into the vector at the TOPO<sup>®</sup> site (pCYB5 initiation codon ATG inserted at base pair position 970 of vector).

Sequence	Total size (bp)	<i>AcsI</i> 5'-A(G)/AATTC(T)-3' (bp)
(a) pcDNA3.2/V5/GW/D-TOPO <sup>®</sup> (circular)	5532	128; 1811; 3256
(b) porcine CYB5 cDNA (linear)	470*	48; 116
(c) vector with pCYB5 insert (circular)	6002	128; 1087; 1155; 2281; 3726

\* Porcine cDNA spanning the ATG initiation site at position 78 and the TAA termination site at position 482 of the transcribed mRNA (1724 bp). Sixty-six additional base pairs were PCR amplified downstream of the termination codon in the mRNA nucleotide sequence.

Figure 4-4 shows the calculated base pairs of DNA products as a result of *AcsI* endonuclease digestion of plasmid pCYB5. Five digest sites yield DNA fragments of 68, 959, 1126, 1445 and 2404 bp.

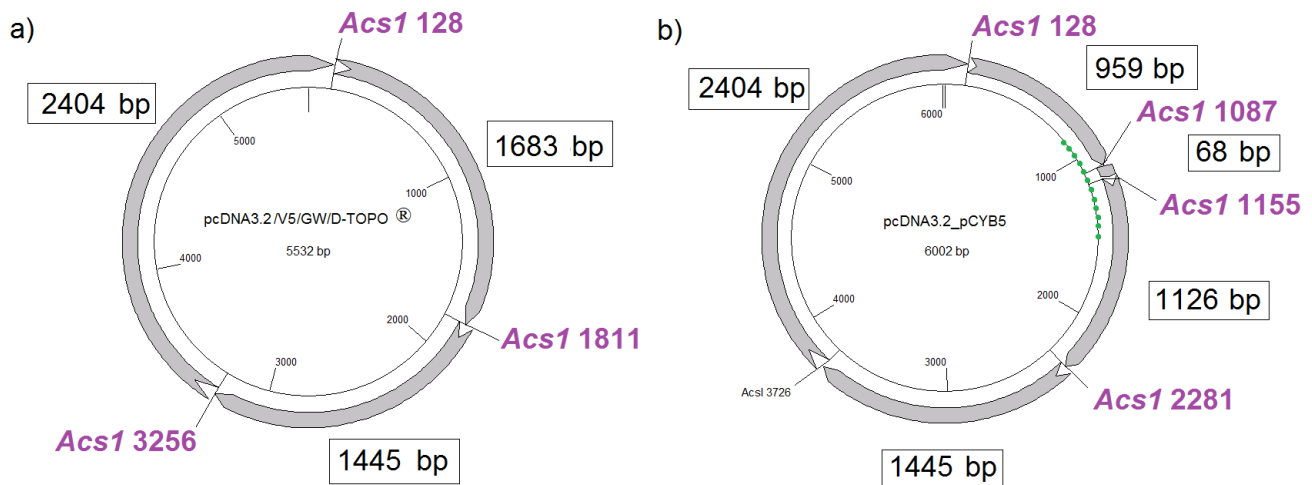


Figure 4-4: Vector diagram of (a) the pcDNA3.2/V5/GW/D-TOPO<sup>®</sup> vector without and b) with the porcine CYB5 insert (green broken line indicates the position of insertion in the vector). Vector, 5532 bp, has three *AcsI* recognition sites at base pair positions 128, 1811 and 3256. The vector containing DNA insert, 6002 bp, has two additional *AcsI* recognition sites. Plasmid digestion by *AcsI* yields estimated DNA fragments of 68, 959, 1126, 1445 and 2404 bp.

Figure 4-5 shows the analyses of isolated plasmids and DNA products resulting from *AcsI* digestion, using gel electrophoresis on a 1% agarose. The 1 kb marker ranges from 250 bp to 10 000 bp.

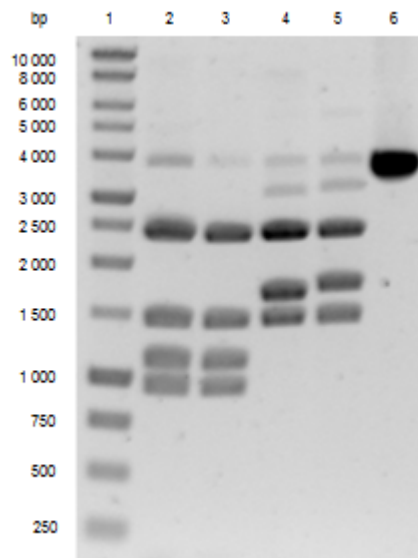


Figure 4-5: Restriction digest of isolated plasmids. Lane 1, 1 kb DNA marker (Roche); lanes 2 and 3, *AcsI* digest of positive clones, pcDNA3.2\_pCYB5; lanes 4 and 5, *AcsI* digest of clones containing vector only; and lane 6, undigested plasmid cDNA. Fragments were separated by gel electrophoresis using a 1% agarose, stained with ethidium bromide for UV visualization.

Lanes 2 and 3 show DNA fragments with sizes that were calculated for the *Acs1* digest of the circular vector containing the porcine CYB5 cDNA insert. Sequence analysis, confirmed that the DNA sequence of clones 1 and 2 were identical to porcine CYB5 (GenBank protein identification number P00172.3).

Table 4-3 shows the absorbance values obtained from UV spectrophotometry analysis of isolated porcine liver mRNA and porcine CYB5 (pCYB5) DNA. Concentration and purity of DNA was calculated via OD measurements at 260 and 280 nm. OD values ranged between 0.1 and 0.8 for optimal measurement. mRNA and DNA yield and concentration were calculated for 50  $\mu$ l and 200  $\mu$ l sample volumes, respectively, and 1  $A_{260}$  Unit of ssDNA = 33  $\mu$ g/ml H<sub>2</sub>O and 1  $A_{260}$  Unit of dsDNA = 50  $\mu$ g/ml H<sub>2</sub>O, respectively. Purity was determined by  $A_{260}/A_{280} \geq 1.6$ .

Table 4-3: Absorbance values of (a) isolated porcine liver mRNA and (b) purified pCYB5 plasmid DNA.

Isolated	absorbance values		dilution	concentration	dsDNA	purity
	260 nm	280 nm	factor	( $\mu$ g/ $\mu$ l)	( $\mu$ g)	260:280
a) mRNA <sub>1</sub>	0.65	0.39	40	0.86	42.9	1.66
mRNA <sub>2</sub>	0.46	0.29	40	0.59	29.7	1.59
b) pCYB5 <sub>1</sub>	0.72	0.46	100	3.61	722	1.59
pCYB5 <sub>2</sub>	0.72	0.46	100	3.60	720	1.56

## 4.5.2 Analyses of prog conversion by CYP17A1

### 4.5.2.1 Prog conversion by wt pCYP17A1-VH co-expressed with different species of CYB5

The expression of wt pCYP17A1-VH (0.25  $\mu$ g) in COS-1 cells without CYB5 resulted in prog conversion yielding 80.2 % 17OHprog and 16.4% A4, with negligible (3.4%) substrate remaining (see Figure 4-6 and Table 4-4). In the presence of equimolar ratios of pCYB5 (0.25  $\mu$ g), the catalytic activity of wt pCYP17A1-VH was significantly influenced, shifting the formation of A4 to 85.6%, with only 13.0% 17OHprog remaining. It has previously been shown that co-transfecting CYP17A1 and CYB5 at a 1:1 ratio (0.25  $\mu$ g plasmid of each) achieves optimal catalytic conditions.

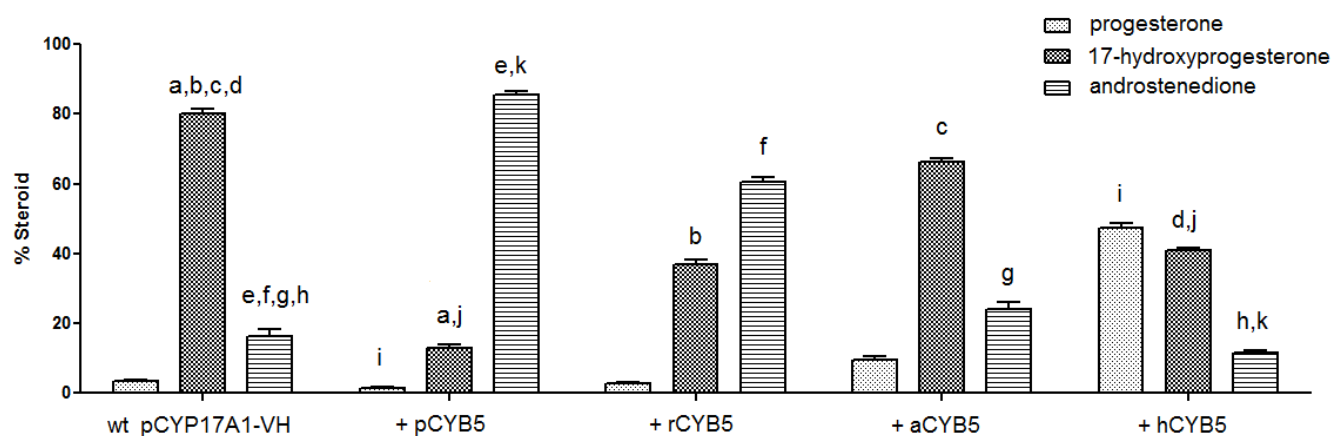


Figure 4-6: Steroid profiles of 1  $\mu$ M prog conversion after 24 hr by wt porcine CYP17A1 (wt pCYP17A1-VH) without and in the presence of different species CYB5: porcine (pCYB5), rat (rCYB5), angora (aCYB5) and human (hCYB5), expressed in COS-1 cells. Results are representative of three independent experiments (n = 9). Individual steroids were compared, represented by matching alphabetical *letters*, by a one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean  $\pm$ SEM. Comparisons of 17OHprog (a,b,c,d) formation between wt and wt in the presence of porcine- (a), rat- (b), angora- (c) and human (d) CYB5, respectively, as well as comparisons of A4 (e,f) production between wt and wt in the presence of porcine- (e) and rat (f) CYB5, respectively, were significantly ( $p < 0.0001$ ) different from each other. Comparison of A4 (g) formation between wt and wt with angora CYB5, was also significantly different with  $p = 0.0480$ . Human CYB5 significantly ( $p < 0.0001$ ) reduced wt pCYP17A1 17 $\alpha$ -hydroxylase and 17OHprog (d) formation, with the concomitant and significantly ( $p < 0.0001$ ) higher levels of prog (i) remaining. Comparisons between A4 (h) formation by wt and wt in the presence of human CYB5, were not significantly different to each other. Comparisons between porcine CYB5 and human CYB5 activities and their effects on wt pCYP17A1 17OHprog (j) and A4 (k) formation, were significantly ( $p < 0.0001$ ) different to each other.

Co-expression of porcine CYP17A1 with CYB5 from a species other than porcine, did not enhance porcine CYP17A1 17,20-lyase activity to the degree that porcine CYB5 did. Comparisons of steroid product formation by wt porcine CYP17A1 expressed without and with different species CYB5 clearly shows (Table 4-4) that while pCYB5 enhanced the lyase reaction and increased the A4 production 5.2-fold, the effect on the lyase activity in the presence of different species CYB5 is less favourable. A lesser increase is shown in the production of A4 by rat CYB5, rCYB5 (3.7-fold) and angora CYB5, aCYB5 (1.5-fold) In the presence of human CYB5, hCYB5, although not significant, produced less A4 (1.4-fold). In addition, expression of CYP17A1 in the presence of human CYB5 also influenced the hydroxylase activity and after 24 hr, nearly half the substrate remained (47.5%). with 2-fold less 17 $\alpha$ -hydroxylated product than in wt.

As CYB5 influences 17,20-lyase activity, ratios of A4:17OHprog+A4 obtained in the conversion of prog by porcine CYP17A1 in the presence of the different CYB5 were determined (Table 4-4).



Table 4-4: Percentage steroids present after 24 hr of 1  $\mu$ M prog conversion and ratios of 17,20-lyase:17 $\alpha$ -hydroxylase products after wt pCYP17A1-VH expression in the presence of CYB5 from different species.

	prog %	17OHprog%	A4%	A4:17OHprog+A4
wt pCYP17A1-VH	3.4	80.2	16.4	0.17
+ pCYB5	1.4	13.0	85.6	0.87
+ rCYB5	2.6	36.8	60.6	0.62
+ aCYB5	9.5	66.4	24.1	0.27
+ hCYB5	47.5	40.9	11.6	0.22

Porcine CYB5 affects porcine 17,20-lyase activity in that the production of A4 is more efficient (5.2-fold increase) than rat- (3.7-fold increase), angora- (1.5-fold increase) and human CYB5 (1.4-fold reduction) A4 production. It is quite apparent that same species co-expression achieves optimal activity.

#### 4.5.2.2 Analyses of 16OHprog production by wt pCYP17A1-VH in the presence of pCYB5 and rCYB5

16 $\alpha$ -Hydroxylase activity was investigated by co-expressing wt pCYP17A1-VH, pCYB5 and rCYB5. Figure 4-7 shows the steroid profiles, analysed by UPLC-MS, for the prog conversion assays after 24 hr.

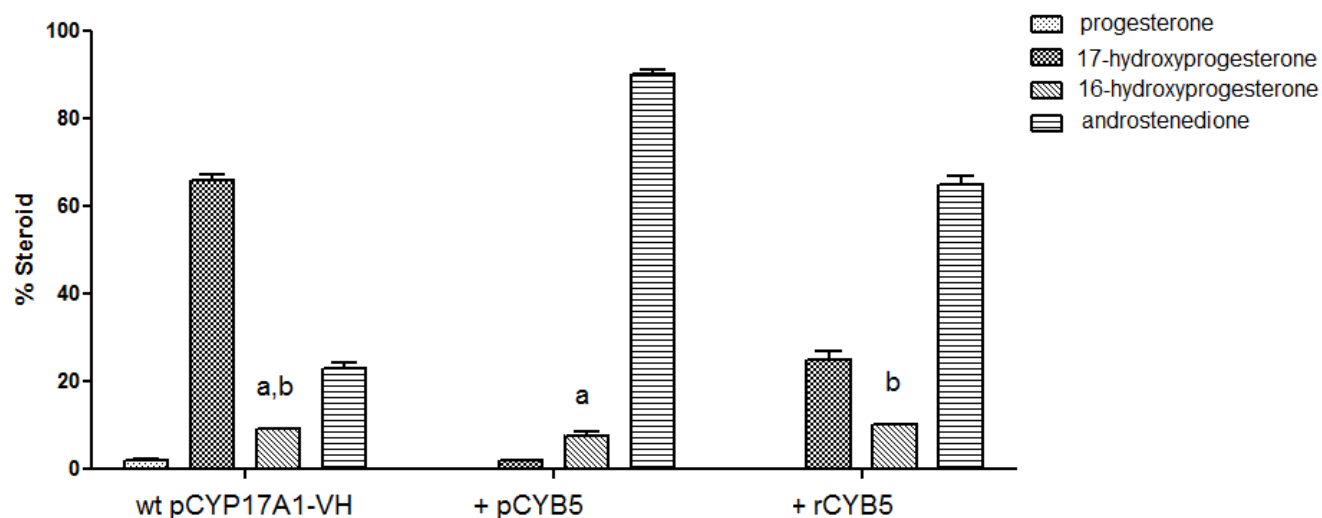


Figure 4-7: Effect of CYB5 on 16OHprog formation after 24 hr. Results are representative of three independent experiments (n = 9). Individual steroids were compared by a one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean  $\pm$ SEM. Comparisons of 16OHprog formation between wt without and in the presence of porcine- (a) or rat CYB5 (b) were not significantly different to each other.



Levels of 16OHprog were negligible, neither porcine CYB5 nor rat CYB5 influences the formation of this steroid (Table 4-5). The change in ratio of products formed via C17 hydroxylation and C16 hydroxylation are not significantly different.

Table 4-5: Analyses of CYB5 influence on 16 $\alpha$ -hydroxylase activity of wt pCYP17A1-VH.

Plasmid cDNA	prog %	17OHprog %	16OHprog %	A4 %	16OHprog:17OHprog+A4
wt pCYP17A1-VH	1.9	66.1	9.1	22.9	0.10
+ pCYB5	0.0	2.0	7.7	90.3	0.08
+ rCYB5	0.0	24.8	10.1	65.1	0.11

#### 4.5.2.3 Analyses of prog conversion by wt pCYP17A1-VH and mutant pCYP17A1-LL co-expressed with pCYB5

pCYP17A1-LL, is a double mutant of wt pCYP17A1, containing the Val40Leu and His407Leu substitutions. In the absence of CYB5, they have quite different activities (Figure 4-8).

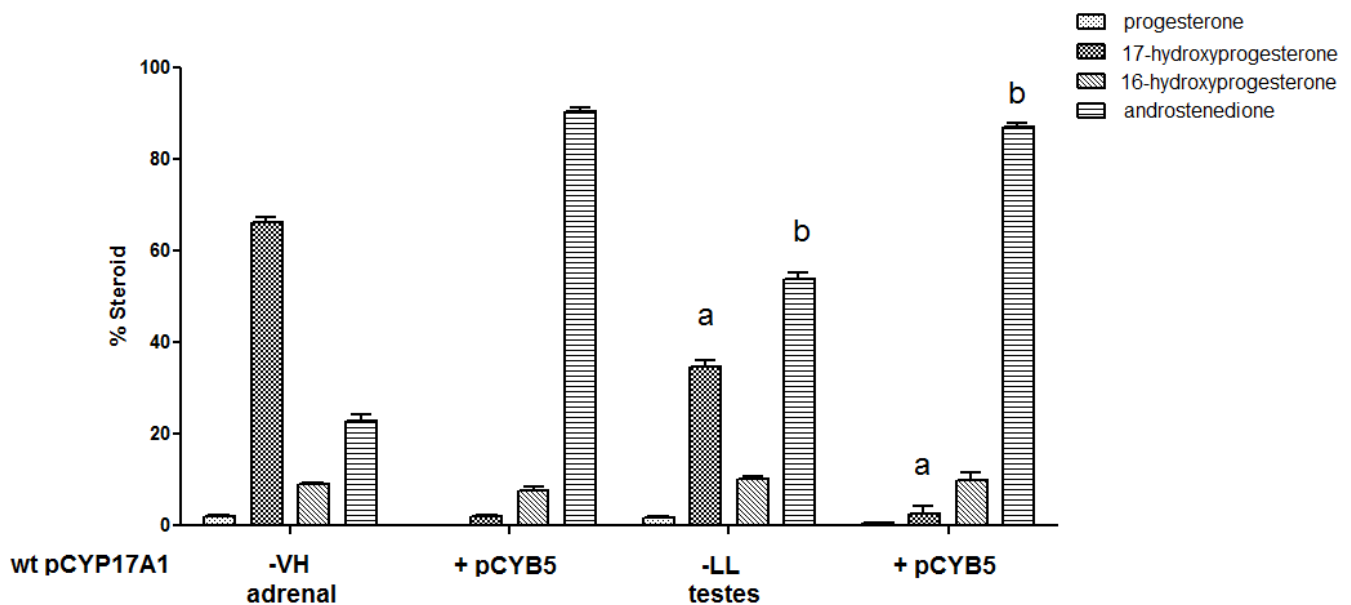


Figure 4-8: Similar steroid profiles at 24 hr after wt (Val40/His407) and double mutant pCYP17A1-LL (Leu40/Leu407) amplification by pCYB5. As seen in wt, there is a significant change ( $p < 0.0001$ ) in both 17OHprog (a) and A4 (b) production upon addition of pCYB5 with mutant pCYP17A1-LL. There are no significant differences in steroid product percentages between the co-expression steroid profiles of wt+pCYB5 and LL+pCYB5.

Compared to wt ratio (0.74), see Table 4-6, the double mutation of pCYP17A1-LL favours the catalysis of the lyase reaction and has a ratio of 0.39 for products formed via C17 hydroxylation and C17,20-lyase in the total products formed. Interestingly, the effect of pCYB5 on the lyase reaction is more prominent in the wt pCYP17A1-VH conversion of prog, which results in a 3.9-fold increase in A4 production, when compared to the 1.6-fold increase in the case of the double mutant.

Table 4-6: Steroid analyses of prog conversion by double mutant pCYP17A1-LL and wt pCYP17A1-VH in the presence of, and without CYB5.

Plasmid cDNA	prog %	17OHprog %	16OHprog %	A4 %	17OHprog:17OHprog+A4
wt pCYP17A1-VH	1.9	66.1	9.1	22.9	0.74
+ pCYB5	0.0	2.0	7.7	90.3	0.02
pCYP17A1-LL	1.6	34.6	10.2	53.6	0.39
+ pCYB5	0.6	2.6	9.9	86.9	0.03

Wt pCYP17A1-VH (Val40/His407) differs from porcine testes CYP17A1 (Leu40/Leu407) at two positions. Wt double mutant -LL comprise both V40L and H407L substitutions (Leu40/Leu407); thus representative of the testicular protein. pCYP17A1-LL produces higher levels of A4 than wt pCYP17A1-VH, as is reflected in the ratios 0.61 and 0.26, respectively, regardless of the presence of CYB5, a 2.3-fold difference in formed A4.

The production of 16OHprog remained negligible and unchanged for both wt and -LL, regardless of the presence of pCYB5.

#### 4.6 Discussion

In this study, pCYB5 was cloned in order to investigate the effect of pCYB5 on A4 production by porcine CYP17A1. Other species CYB5 were included in the study to determine whether optimal activity was achieved when expressing wt pCYP17A1 with human-, rat- and angora CYB5. It was found that co-transfecting CYP17A1 and CYB5 at a 1:1 ratio achieved

After 24 hr, the co-expression of wt pCYP17A1-VH with the four species CYB5s resulted in significant differences in steroid profiles, although no significant changes were observed in 16 $\alpha$ -hydroxylase activity. The greatest stimulation of the lyase reaction was obtained when porcine CYP17A1 was expressed with porcine CYB5 and a 6-fold increase in A4 levels was measured, while

rat CYB5, on the other hand, yielded a 2-fold increase in A4 levels. The conversion of prog by wt pCYP17A1-VH in the presence of angora CYB5 yielded significantly less A4 than in the presence of porcine and rat CYB5; with a concomitant increase in 17OHprog levels. While the A4 produced in the presence of angora CYB5 was also significantly less than the levels detected in the presence of porcine CYB5, it was nevertheless more than that produced by wt in the absence of CYB5. In contrast, human CYB5 not only resulted in significantly lower A4 levels but also hampered the hydroxylase activity.

The CYB5 and CYP17A1 gene sequences comprise 402 bp and 1786 bp nucleotides, respectively, which encode the 134- and 508 amino acid proteins, respectively. Interactions between specific residues of same species CYP17A1 and CYB5 are dependent on the conformation of the proteins within the enzyme/redox partner complex, which probably allow for species-specific optimal enzyme activity. Single residues or mutations have been attributed to CYB5 potential to maximally amplify/reduce CYP17A1 activity, as well as being responsible for appropriate interaction between the heme proteins. Although the amino acids in the P450 substrate binding site define the substrate specificity of the individual forms of P450, other undefined regions on the P450 surface are also functionally active as they interact with NADPH-CPR and CYB5 (Omata *et al.*, 1994).

Sequence alignment and analysis shows that although porcine, rat and angora CYB5 share a high degree of sequence identity; their redox partner CYP17A1 sequences do not (Table 4-7).

Table 4-7: Amino acid residue differences (number) and percentage identities (in parenthesis) between porcine CYB5 and CYP17A1 and the human, rat and angora homologs.

		human	rat	angora
CYB5 (134 residues)	porcine	18 (86.5%)	11(91.7%)	13 (90.2%)
CYP17A1 (508 residues)	porcine	173(66.0%)	393 (22.7%)	111 (78.1%)

An alignment of various species CYB5 (Addendum C), shows that there is a conserved region of amino acid residues, ~35-74, with variations occurring near the N- and C- terminals (Hagihara and Furuya, 1978). Secondary structural features obtained for a few of the species CYB5 (see Figure 4-1) show that in the heme-binding domain (amino acid residues ~60-80), residues His44 and His68 co-ordinate heme positioning (Aono *et al.*, 2010), and residues Glu48, Glu49, Glu64, Asp65, Val66, Thr70-Ser76 are shown to specifically interact with P450 residues in the CYB5-P450 interface (Ahuja *et al.*, 2013). These residues appear to be conserved for current known sequences of mammalian CYB5. The alignment of the species CYB5 investigated in this study (Figure 4-3); show the same residues in these

structural domains, with all of the conserved residues being identical with published data (Musket *et al.*, 1996; Arnesano *et al.*, 1998; Banci *et al.*, 2000; Nunez-Quintana *et al.*, 2010, Ahuja *et al.*, 2013).

In accordance with the literature, cloned porcine CYB5 amplified the production of A4 by wt pCYP17A1-VH, which ranged from 16.4% without CYB5 to 85.6% with CYB5, with no apparent influence on hydroxylase activity. This mechanism of allosteric behaviour of enhancing 17,20-lyase activity, has been widely reported. It is interesting to note that even though porcine and rat CYP17A1 share the least sequence identity (22.7%) it is rat CYB5 which yields a greater stimulation of the porcine CYP17A1 lyase reaction than did angora and human CYB5, which have far higher CYP17A1 sequence identity. In addition, it is only when human CYB5 forms the redox/partner enzyme complex that there is a negative effect on wt pCYP17A1-VH hydroxylase activity, with only 48% of the substrate being metabolised after 24 hr.

The influence of the sequence identity of CYB5 on the ratio of A4 production by wt porcine CYP17A1 in the presence of and without porcine CYB5, rat CYB5, angora CYB5 and human CYB5 is shown in Figure 4-9.

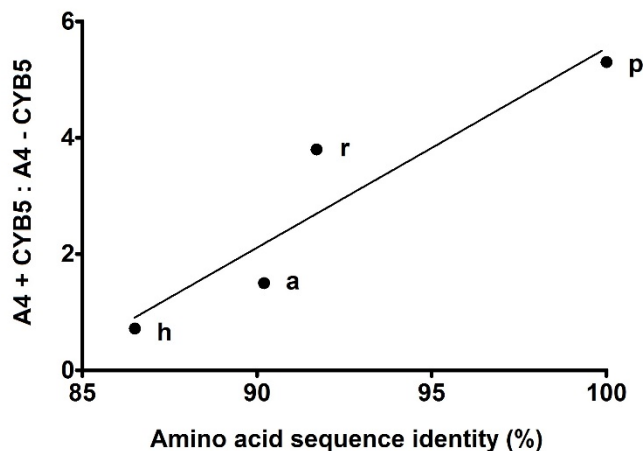


Figure 4-9: Influence of sequence identity on the ratio of A4 production by wt pCYP17A1 in the presence of- and without porcine CYB5 (p), rat CYB5 (r), angora CYB5 (a) and human CYB5 (h).

The linker domain spanning residues Ser90 to Asp104 of CYB5 connects the cytosolic heme domain to the  $\alpha$ -helical transmembrane anchor and it is the linker region that appears to lack distinct structure (Ahuja *et al.*, 2013). Residues within this region, 96 to 103, are essential in the formation of a functional complex between CYB5 and CYP17A1 (Clark *et al.*, 2004). Residues 96 to 103 of porcine

and rat CYB5 are identical. In this region, human has a Pro at position 96 (porcine, rat and angora have a Ser residue) possibly resulting in a bend in the polypeptide chain. Porcine and rat have a Val103 and a Glu104, while human and angora exhibit conservative changes, Ile and Asp, respectively. The influence of human CYB5 on porcine wt CYP17A1 however, cannot be attributed solely to the Pro residue at position 96 with residues in pCYP17A1 also affecting structure and interaction within the enzyme/redox partner complex.

The specificity of CYP17A1 towards its substrates differs in various species and tissues. In human adrenals and testes, CYP17A1 17 $\alpha$ -hydroxylates both preg and prog but selectively produces DHEA in greater quantities than A4 (Chung *et al.*, 1987). CYP17A1 is not expressed in rat or mice adrenal glands (van Weerden *et al.*, 1992) but the gonadal CYP17A1 in these species preferentially produces A4 (Fevold *et al.*, 1989). CYP17A1 is however, expressed in guinea pig (Tremblay *et al.*, 1994) and hamster (LeHoux and Lefebvre, 1980) adrenal glands, producing both DHEA and A4 but favouring A4 production. In this study, porcine CYP17A1 (wt pCYP17A1-VH), with Val40 and His407, catalysed the conversion of prog (>90%) with and without CYB5. The porcine testes pCYP17A1 variant (with sequence including Leu40 and Leu407) was prepared from wt pCYP17A1-VH and is represented by the double mutant construct pCYP17A1-LL, with both Val40Leu and His407Leu substitutions.

With the aim to increase  $\Delta$ 5-lyase activity in hamster, mutation Asp407His replaced the Asp residue (suggested to contribute to high  $\Delta$ 4-lyase activity in small animals) with the His residue (suggested to contribute to high  $\Delta$ 5-lyase activity in larger animals), which substituted a negatively charged side-chain with a positive charge (Mathieu *et al.*, 2002). Activity assays of wt hamster CYP17A1 and mutant Asp407His, in transiently transfected COS-1 cells, show that substrate (2.5  $\mu$ M preg or prog) utilization was linear with time (1 hr). Compared to wt, the Asp407His mutant only showed a selective decrease in 17 $\alpha$ -hydroxylase activity for the  $\Delta$ 4 pathway by 46%, leading to a comparable decrease in A4 formation, which was attributed to the reduced production of 17OHprog. The Trp406Arg mutation in CYP17A1 resulted in two adjacent positive charges (Arg406/His407) in the meander region and led to 17 $\alpha$ -hydroxylase deficiency in prog metabolism (Costa-Santos *et al.*, 2004). In this study, the mutation His407Leu in wt pCYP17A1-VH, which resulted in the loss of a positive charge at the start of the meander region, led to a 2-fold increase in A4 production inferring stimulation of wt lyase activity.

There is modest sequence identity occurring between the ~500 amino acids of various species CYP17A1s and a greater sequence identity occurring between the 134 amino acids of various species CYB5s. Therefore, the aim of this chapter was to observe whether there was a specific relationship or

interaction between same species CYP17A1 and CYB5 in conferring maximal activity by that species CYP17A1 protein. This was achieved by investigating whether or not wt pCYP17A1-VH activity was amplified in a similar manner when co-expressed with alternative species CYB5 cDNA. In comparison with isolated porcine testes CYP17A1 (Leu40 and Leu407) and the partial amino acid sequence of porcine CYP17A1 (Leu40 and His407) derived from adrenal cDNA (GenBank accession numbers [NP\\_999593.1](#) and [AAA84419.1](#) respectively), porcine wt CYP17A1 used in this study, exhibited Val40 and His407 (wt pCYP17A1-VH) and showed typical enzyme characteristics with minimal 17,20-lyase activity. It was rigorously stimulated (6-fold) by porcine CYB5, to a lesser extent by rat CYB5 (above 90% sequence identity) and to even lesser and no extent with angora and human CYB5 (90.2% and 86.5%, respectively). It appears that porcine CYP17A1 with Val40 and His407 was stimulated by porcine CYB5 more intensely than porcine CYP17A1 with Leu40 and Leu407 residues (representing the testes variant).

With regards to how single amino acid substitutions affect- or why other species CYB5 do not similarly affect porcine CYP17A1 activity, speculations that are based solely on end-product formation and steroid profiles are ultimately deemed limited. Therefore, to have a more complete understanding of the effects of the site-directed mutagenesis, future studies would include characterizing wt- and mutant proteins with respect to the rates of their dual catalytic reactions in prog metabolism, with and without the various species CYB5. Furthermore, elaborated 3D models of wt and mutant porcine CYP17A1 with and without various species CYB5, would accurately predict the structural consequences of such mutations and any related restructuring in heme, redox-partner and substrate binding sites.

## CHAPTER 5

### CONCLUSION

The central aim of this study was to investigate the structure/function relationship of CYP17A1, a key enzyme in adrenal and gonad steroidogenesis. Sequence alignments of the deduced amino acid residues of CYP17A1 from various species have allowed for the prediction of ligand binding domains (Auchus and Miller, 1999; DeVore and Scott, 2012), while site-directed mutagenesis and kinetic studies have identified potential active pocket residues (Lin *et al.*, 1991; Monno *et al.*, 1993; Koh *et al.*, 1993; Fardella *et al.*, 1994; Lee-Robichaud *et al.*, 1998; Di Cerbo *et al.*, 2002; Sherbet *et al.*, 2003; Pandey and Miller, 2005; Lin *et al.*, 2005a; Sahakitrungruang *et al.*, 2009; Kok *et al.*, 2010). The homology models of mammalian P450 that are currently available have been generated based mostly on the crystal structures of bacterial enzymes and although they share less than 20% amino acid sequence identity, they share a common structural core even though these proteins differ with regards to solubility, sequence identity, redox partner and substrate specificity (Auchus and Miller, 1999; Kemp *et al.*, 2005). The recent determination of the crystal structures for several of mammalian P450 have enhanced the accuracy of homology modeling. In an effort to promote insight into the structure/function relationship of CYP17A1, the aims of this study were to investigate the catalytic activity of porcine CYP17A1 and the effects of the amino acid residues at positions 40 and 407 on the catalytic activity of porcine CYP17A1; to clone porcine cDNA encoding CYB5 from mRNA isolated from porcine liver and to investigate the influence of CYB5 on the catalytic activity of the enzyme. The influence of porcine CYB5 on the lyase activity of porcine CYP17A1 was subsequently compared to that of human, rat and angora CYB5.

The amino acid sequence of a protein and the reactive side-chain groups determine the manner and frequency in which the protein forms  $\alpha$ -helices and  $\beta$ -sheets and ultimately folds into its 3D structure. It is this unique protein conformation that is responsible for defining the catalytic activity of the enzyme. Changes to one or more of amino acid residues do not necessarily result in changes in the catalytic activity even though they may impact on the conformation of the protein. Changes within the active site, substrate docking site and redox partner binding site will to a great extent influence the ability of an enzyme to function in its typical catalytic manner.



Single base-pair changes in human CYP17A1 cDNA have led to mutations in key amino acid residues, reducing the C17,20-lyase activity. These residues (i.e. Arg347His, Arg358Gln and Arg449Ala) have been well documented and shown to be essential for CYP17A1 interaction with CYB5 (Lee-Robichaud *et al.*, 1998; Lee-Robichaud *et al.*, 1999; Geller *et al.*, 1999). Mutation Trp406Arg abolishes both hydroxylase and lyase activities of human CYP17A1 rendering the enzyme inactive (Costas-Santos *et al.*, 2004). Human *CYP17A1* consists of eight exons, which spans 6.6 kb, is located on chromosome 10q24.3, and is transcribed into the same 2.1 kb mRNA species in the adrenal and gonads, of which 1.6 kb contains coding DNA (Matteson *et al.*, 1986; Chung *et al.*, 1987; Picado-Leonard and Miller, 1987). In general, it is understood that CYP17A1 protein is encoded by a single gene (Miller and Auchus, 2011). However, recent publications have reported that in sheep, a single gene exists as two sequences, which differ by two nucleotides (Storbeck *et al.*, 2008b). Furthermore, two CYP17A1 isoforms were found to be the product of two separate CYP17A1 genes (available on GenBank) encoding two unique CYP17A1 isoforms (Storbeck *et al.*, 2008b) rather than the product of two alleles of the same gene. To date, no SNP's affecting CYP17A1 activities have been detected in the coding region of porcine CYP17A1 cDNA from a variety of different breeds (Yorkshire, Duroc, Landrace, Pietrain) (Lin *et al.*, 2005; Moe *et al.*, 2007; Grindflek *et al.*, 2010).

In this study, the amino acid sequence of porcine wt CYP17A1 (adrenal) differed from the published sequences of porcine adrenal (Chung *et al.*, 1987) and porcine testes (Conley *et al.*, 1992) CYP17A1 cDNA. Sequence analysis of wt identified Val40 and His407 amino acid residues, with the adrenal CYP17A1 protein having Leu40 and His407 residues and the testicular protein having Leu40 and Leu407 residues. Mutant constructs were thus prepared, using wt (Val40 and His407) to include constructs encoding both the porcine adrenal and the testicular protein sequences, pCYP17A1-LH and pCYP17A1-LL, respectively, as well as a mutant incorporating Val40 and Leu407 (pCYP17A1-VL).

Amino acid residues at positions 40 and 407 have not been the focus of earlier investigations in any specie CYP17A1 sequence. Sequence alignments between human and porcine CYP17A1, together with the secondary structural features obtained from the human CYP17A1 crystallized structure (Auchus and Miller, 1999; DeVore and Scott, 2012), showed the residue at position 40 to lie in the A'-helix and the residue at position 407 to lie at the start of the meander region between K'-helix and the highly conserved heme-binding domain. Substitutions at these two positions in wt pCYP17A1-VH – Leu40 and Val40, neutral residues, and His407, a positively charged residue – appear to affect the catalytic activity of the enzyme.



Differences are evident when comparing the levels of 17OHprog and A4, especially in the conversion by the Val40/Leu407 construct. The conversion of prog in the case of Val40/His407 (wt adrenal) and Leu40/His407 (GenBank adrenal) were similar, producing metabolite levels not significantly different from each other. This was expected as they are both adrenal proteins and noteworthy, perhaps, is that a conservative substitution at position 40 had an effect on enzyme activity.

Conversion by Val40/Leu407 and the Leu40/Leu407 constructs resulted in significantly higher A4 levels with conversion by Val40/Leu407 showing a steroid profile significantly different ( $p < 0.0001$ ) to that of wt, as well as the two other constructs. In the conversion of prog by Val40/Leu407, the levels of A4 produced was 3.9-fold more than that produced by wt –Val40/His407, with a concomitant decrease of 23.6-fold in the level of 17OHProg, clearly indicating that the His residue at position 407, resulted in a decrease in the lyase activity. Interestingly, compared to wt, a 2.3-fold increase in A4 was detected in the conversion by the construct encoding the published testicular Leu40/Leu407 protein sequence, which is corroborated by reports that porcine testes has a high potential for C17,20-cleaved steroids. It is also interesting to note that the testicular construct (encoding Leu40/Leu407 residues) produced significantly more A4 than the construct encoding the published sequence for the adrenal protein (encoding Leu40/His407 residues), which is also corroborated by reports that although porcine adrenal is capable of  $\Delta 4$  androgen production, more is produced by the testes. This tissue specific activity, which according to observations obtained in this study, could possibly be related to the single amino acid differences shown between the published sequences of adrenal and testes CYP17A1. However, the possibility of either the same gene existing as two sequences (as was found in sheep) or two distinct genes (as was found in angora) encoding porcine CYP17A1 adrenal and testes proteins would require further investigation.

Subsequent analyses of the data also showed that there were no significant differences in 16OHprog production, underlying the findings that these substitutions at positions 40 and 407 did not influence the hydroxylase activities. In addition, these data also suggest that, while maintaining the Leu residue at position 407, mutating the Leu40 residue of porcine testicular CYP17A1 to a Val residue led to a significant increase in A4 production. Although this is a conservative change, the bulkier side chain of Leu in the A'-helix may influence the conformation of the protein with the shorter branched chain of Val leading to a more favourable positioning of the substrate and subsequent interaction with the redox partners.

Substitutions at these two positions in wt pCYP17A1, affected predominantly the lyase activity of the enzyme. Comparing the sequences of porcine and human CYP17A1, identified a His407 residue in the human protein, while also showing that the porcine sequence has a Leu residue at position 105. Human CYP17A1 has an Ala105 residue, which is associated with the 16 $\alpha$ -hydroxylase activity. Although porcine CYP17A1 catalyses 16 $\alpha$ -hydroxylation reactions, 16OHprog formation is relatively low (~10%) in comparison with 17OHprog (~70%) and A4 (~20%) formation, therefore the mutation Leu105Ala was constructed (pCYP17A1-LA) to investigate associated changes in 16 $\alpha$ -hydroxylase activity. This mutant decreased the overall catalytic activity of expressed enzyme, with 71.7% prog substrate remaining after 24 hr, a 5-fold decrease when compared to wt. Interestingly, there was a significant change in the ratio of 16OHprog:17OHprog+A4 from 0.10 for the wt to 0.57 for the mutant indicating that the increased change in this ratio was due to the mutation itself and that it was only the 17 $\alpha$ -hydroxylase activity, which was influenced by the mutation and not the 16 $\alpha$ -hydroxylase activity.

In the human adrenal, CYP17A1 produces significant levels of 16OHprog. Prog metabolism was assayed in COS-1 cells transiently transfected with wt human CYP17A1, which produced a 17OHprog:16OHprog ratio of ~3:1, regardless of the presence of CYB5. The mutation Ala105Leu resulted in the production of negligible 16OHprog levels, with no apparent influence on 17 $\alpha$ -hydroxylase activity, regardless of the presence of CYB5 (Swart *et al.*, 2010). Co-expression with CYB5 significantly decreased the ratio of 17OHprog:16OHprog in the proteins with Leu105, while effects were negligible with Ala at this position (Swart *et al.*, 2010). Baboon CYP17A1 (Leu105) with mutation, Leu105Ala, exhibited the same 17OHprog:16OHprog ratio (~3:1) observed in wt human CYP17A1 (His407); although the rate of prog conversion was reduced. In this study, the Leu105Ala mutation in wt pCYP17A1 (His407) showed to significantly reduce wt 17OHprog formation. Homology models showed that Ala105 faced towards the active pocket in the predicted B'-C domain of CYP17A1 (Swart *et al.*, 2010). It was suggested that this smaller residue allows more flexibility of movement in the active pocket than Leu does, presenting both the C16 and C17 of prog to the iron-oxygen complex. With regards to species investigated for 16 $\alpha$ -hydroxylase activity, human CYP17A1 is the only protein with a His residue at position 407 and although porcine CYP17A1 (Chung *et al.*, 1987) and porcine CYP17A1 assayed in this study, have a His residue at position 407, both exhibit low 16 $\alpha$ -hydroxylase activity with the 16OHprog produced being negligible. The low levels could not be quantified accurately as these fall below the lowest levels of quantification of the analytical methods, which were used.

Different species of CYB5, sharing high degrees of amino acid sequence identity, were co-expressed with wt pCYP17A1 in COS-1 cells to investigate the influence of CYB5 on the catalytic activity of porcine CYP17A1. Porcine CYB5 was also co-expressed with the double mutant pCYP17A1-LL (representing the testicular protein). The expression of wt pCYP17A1-VH in COS-1 cells without CYB5 resulted in prog conversion yielding 80.2 % 17OHprog and 16.4% A4, with negligible (3.4%) substrate remaining. In the presence of equimolar ratios of pCYB5, the catalytic activity of wt pCYP17A1-VH was significantly influenced, shifting the formation of A4 to 85.6%, with only 13.0% 17OHprog remaining. Co-expression of porcine CYP17A1 with CYB5 from a species other than porcine, did not enhance porcine CYP17A1 17,20-lyase activity to the degree that porcine CYB5 did. Comparisons of steroid product formation by wt porcine CYP17A1 expressed without and with different species CYB5 clearly shows that while porcine CYB5 enhanced the lyase reaction and increased the A4 production 5.2-fold, the effect on the lyase activity in the presence of different species CYB5 was less favourable. A smaller increase is shown in the production of A4 by rat CYB5 (3.7-fold) and angora CYB5 (1.5-fold). In the presence of human CYB5, lower levels of A4 were detected than was produced by wt pCYP17A1 in the absence of CYB5, although not significant. In addition, expression of wt pCYP17A1 in the presence of human CYB5 also influenced the hydroxylase activity and after 24 hr, nearly half the substrate remained (47.5%). with 2-fold less 17 $\alpha$ -hydroxylated product being produced in comparison to wt pCYP17A1 in the absence of CYB5. As CYB5 influences 17,20-lyase activity, ratios of A4:17OHprog+A4 obtained in the conversion of prog by porcine CYP17A1 in the presence of the different CYB5 were determined. Porcine CYB5 affects porcine 17,20-lyase activity in the production of A4 and is more efficient (5.2-fold increase) than rat CYB5 (3.7-fold increase), angora CYB5 (1.5-fold increase) and human CYB5 (1.4-fold reduction) in stimulating A4 production. It is quite apparent that same species co-expression achieves optimal activity. It would, therefore, appear that while optimal lyase conversion may be achieved by co-expression of CYP17A1 with the same species CYB5, expression in the presence of CYB5 of different species may impede the conversion of the substrate in that the hydroxylase reaction is affected.

The 16 $\alpha$ -hydroxylase activity was subsequently investigated by co-expressing wt pCYP17A1-VH with porcine CYB5 and rat CYB5. Levels of 16OHprog were negligible, with neither porcine nor rat CYB5 influencing the formation of this steroid, as the change in ratio of products formed via C17 hydroxylation and C16 hydroxylation were not significantly different. It is, however, possible that if wt pCYP17A1-VH was co-expressed with human CYB5, the 16OHprog levels may have been reduced, indicating that human CYB5 hinders the hydroxylase activity of CYP17A1. To investigate the effect of

CYB5 on CYP17A1 lyase activity, wt pCYP17A1-VH and pCYP17A1-LL (containing both Val40Leu and His407Leu substitutions), were co-expressed with porcine CYB5. In the absence of CYB5, they have quite different activities with pCYP17A1-LL favouring the catalysis of the lyase reaction with a ratio of 0.39 for products formed via C17 hydroxylation and C17,20- lyase while a 0.74 ratio was obtained with wt pCYP17A1. Interestingly, the effect of porcine CYB5 on the lyase reaction is more prominent in the wt pCYP17A1-VH conversion of prog, which results in a 3.9-fold increase in A4 production, when compared to the 1.6-fold increase in the case of pCYP17A1-LL. Porcine CYP17A1-LL, representing testicular CYP17A1, produces 2.3-fold more A4 than wt pCYP17A1-VH (adrenal), regardless of the presence of CYB5, suggesting the possible significance of residues at position 40 and/or 407. The production of 16OHprog remained negligible and unchanged for both wt and -LL, regardless of the presence of pCYB5.

CYP17A1 exhibits a dual catalytic activity in terms of its hydroxylase and lyase activities, with the enzyme being species-specific regarding the preg and prog substrates, and the hydroxylated intermediates. Numerous studies have been conducted investigating the preferred pathway for porcine androgen production via  $\Delta 5$  CYP17A1 activity. Furthermore, contributions towards the characterisation of these activities have been achieved by clinical cases describing mutations, as well as mutations engineered by site-directed mutagenesis, which influence the enzymes behaviour.

Residues at position 407 in CYP17A1 from most small laboratory animals (species with preferred  $\Delta 4$  lyase activity) are Asp407 in hamster and guinea pig (adrenal and testes enzymes) and Gln407 in rat and mouse (testicular protein only). Adrenal and testes CYP17A1 from larger animals (species with preferred  $\Delta 5$  lyase activity) have residues His407 in human, His/Leu407 in porcine, respectively, and Gln407 in bovine, angora and sheep (Miller and Auchus, 2011). In human testes, enzyme kinetics data indicate a >10-fold higher preference for testosterone formation via the  $\Delta 5$  pathway than the  $\Delta 4$  pathway (Fluck *et al.*, 2003). Production of A4, by expressed wt pCYP17A1-VH and -LL (porcine testes), both increased to ~90 % in the presence of CYB5. Accurate stimulation of the lyase reaction by wt pCYP17A1-VH and pCYP17A1-LL can only be assessed once the catalytic parameters have been determined. However, conclusions from studies relating CYP17A1 activity to amino acid residues in a protein sequence, allow for inferences regarding the activity of proteins with high acid sequence identity.

Positively charged residues on the proximal face of CYP17A1 generally contribute to the lyase activity as it has been shown that substitutions of these basic proteins result in a loss of lyase activity; but not

the hydroxylase activity. Mutations which neutralize these positively charged residues cause the characteristic loss in 17,20-lyase activity. It is, however, not only surface residues that influence catalytic activity. The Phe417Cys mutation, which resulted in the loss of lyase activity, does not lie at the protein surface but behind a loop between the meander region and the heme binding region; which suggests that changes to the structure of the protein affects the redox partner binding site (Biaison-Lauber *et al.*, 1997).

The addition of CYB5 may have also influenced the catalytic rates of the CYP17A1 reactions; however, in this study, only the end-products were analysed. Future studies would include investigating the effects of the substitutions at positions 40 and 407, as well as the influence of various species CYB5 on the rate of porcine CYP17A1 catalysis by determining the apparent Michaelis constant ( $K_m$ ) and maximum velocities ( $V_{max}$ ) for the dual reactions.

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## ADDENDUM A

Sequence alignment of the 510 amino acids of various species CYP17A1 enzymes relative to the human form. GenBank accession numbers: human (*Homo sapiens*) [NP\\_000093.1](#); cape baboon (*Papio ursinus*) [AAG10599.1](#) and yellow baboon (*Papio cynocephalus*) [Q8HYN0.1](#); bison (*Bison bison*) [Q9GMC7.1](#); bovine (*Bos taurus*) [NP\\_776729.1](#); chimpanzee (*Pan troglodyte*) [Q8HYN1.1](#); angora goat (*Capra hircus*) [ABQ12616.1](#) (ACS-: Ala6, Pro41, Val213) and [ABQ12617.1](#) (ACS+: Gly6, Leu41, Ile213); crab-eating macaque (*Macaca fascicularis*) [ABB76808.1](#) and rhesus macaque (*Macaca mulatta*) [Q8HYM9.1](#); porcine (*Sus scrofa*) [AAA84419.1](#) (His407) and [NP\\_999593.1](#) (Leu407); sheep (*Ovis aries*) [NP\\_001009483.1](#), black bear (*Ursus thibetanus japonicas*) [BAH82843.1](#); cat (*Felis catus*) [NP\\_001009371.1](#); guinea pig (*Cavia porcellus*) [Q64410](#); horse (*Equus caballus*) [BAA13551.1](#); hamster (*Mesocricetus auratus*) [NP\\_001268860.1](#); house mouse (*Mus musculus*) [NP\\_031835.3](#) and white-footed mouse (*Peromyscus leucopus*) [Q91Z85.1](#), and brown rat (*Rattus norvegicus*) [NP\\_036885.1](#).

	10	20	30	40	50	60
human	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	MWELVALLLLT	LAYLFWPKRRC	PGAKYPKSLLS	LPLVGS	LPLPRHGHMHNNEFKLQKKY
baboon, cape	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
baboon, yellow	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
bison	..L.L.VF.....	.....TKHS.....	.....R..P.....	.....R..QQ.K.....	.....E..	.....
bovine	..L.L.VF.....	.....TKHS.....	.....R..P.....	.....R..QQ.K.....	.....E..	.....
chimpanzee	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
goat, angora (ACS-)	..V.L.VF.....	.....TKHSA.....	.....R..P.....	.....R..QQ.E.....	.....E..	.....
goat, angora (ACS+)	..V.LGVF.....	.....TKHSA.....	.....R..P.....	.....L.....R..QQ.E.....	.....E..	.....
macaque, crab-eating	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
macaque, rhesus	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
porcine (H407)	..V.LVFF....T.....	.....TKGS.....	.....R..PV..V.....	.....R..Q.M.....	.....D..	.....
porcine (L407)	..V.LVFF....T.....	.....TKGS.....	.....R..PV..V.....	.....R..Q.M.....	.....D..	.....
sheep	..V.L.VF.....	.....TKHS.....	.....R..P.....	.....R..QQ.E.....	.....E..	.....
bear, black	....L.F.F....FL..	PK.IS.....	.....P.....	.....G..Q.V.....	.....E..	.....
cat	....LVE..FAV..FL..	AK.....	.....P.....	.....L.....S..P.K.....	.....	.....
guinea pig	....T..G.I.....	RQGSS.T.....	.....P..V.....	.....KS.....V.....	.....	.....
horse	....L.F..AI..F.R..	VK.....	.....PY.....	.....P.V.....	.....	.....
hamster	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
mouse, house	....G...I...F....	SKT.N..F.R..	PF.....	.....R...A.....	.....E..	.....
mouse, white-foot	....C...I...F....	PKTSD..F.R..	PF.....	.....V.....	.....E..	.....
rat, brown	....G...I...F..V.	SKT.....	L.R..P.....	.....R...V.....	.....E..	.....

	70	80	90	100	110	120
human	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	GPIYSVRMGTKTT	VIVGHQLAKEVLI	KKGKDFSGR	PQMATLDIASNNR	KGIAFADSGAH
baboon, cape	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
baboon, yellow	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
bison	....F.L.S....MI.....	.....R...L...E.....	.....KV.....	.....L.D.Q.....	.....H..	.....
bovine	....F.L.S....MI.....	.....R...L...E.....	.....KV.....	.....L.D.Q.....	.....H..	.....
chimpanzee	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
goat, angora (ACS-)	....F.L.S....MI.....	.....R...L...E.....	.....KV.....	.....L.D.Q.....	.....H..	.....
goat, angora (ACS+)	....F.L.S....MI.....	.....R...L...E.....	.....KV.....	.....L.D.Q.....	.....H..	.....
macaque, crab-eating	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
macaque, rhesus	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....	.....	.....	.....	.....	.....
porcine (H407)	....F.F.L.S....VI.D.....	.....L...E.....	.....RVM.....	.....L.D.Q.....	.....H.TS	.....
porcine (L407)	....F.F.L.S....VI.D.....	.....L...E.....	.....RVM.....	.....L.D.Q.....	.....H.TS	.....
sheep	....F.L.S....MI.....	.....R...L...E.....	.....KV.....	.....L.D.Q.....	.....H..	.....
bear, black	....L.....M.....	.....V...E.....	.....VV.....	.....L.D.Q.....	.....H..N	.....
cat	....F.L.....M..D.....	.....V...E.....	.....HVV.....	.....L.D.Q.....	.....H..S	.....
guinea pig	....F.L.ST...VI.....	.....R.L...E.....	.....LTT.VALL.D.G.....	.....S.T	.....	.....
horse	....L.....L...Y.....	.....E.....	.....V...N.L.D.Q.V.....	.....H..P	.....	.....
hamster	....L.L.ST...I.QY.....	.....V...E.....	.....H.V..GLL.DQG.....	.....GS	.....	.....
mouse, house	....L.L..T.A....Y...R...V...E.....	.....V...GLL.DQG..V.....	.....SSS	.....	.....	.....
mouse, white-foot	....L.L..IP...I.QY...R...E.....	.....V...GLL.OG.....	.....DDA	.....	.....	.....
rat, brown	....L.L..T...I..Y...R...E.....	.....V.QSLL.DQG..V.....	.....A.SS	.....	.....	.....



	130	140	150	160	170	180
human	WQLHRR	LAMATF	ALFKDGD	QKLEKI	ICQEIST	LCMDLATH
baboon, cape						NGQSIDI
baboon, yellow						SF PVFVAV
bison						TNVNIS
bovine						T.....I.....
chimpanzee						T.....I.....
goat, angora (ACS-)						K..LNA.....NL.....N..ANV...F...QH..EA..L.E.LSL...I..
goat, angora (ACS+)						K..VLNA.....NL.....N..ANV...F...QH.....L.E.LSL...I..
macaque, crab-eating						T.....I.....
macaque, rhesus						T.....I.....
porcine (H407)						K..LS..S...G.NL...N..N...KV...F...R..E...LAQ.LSL.M..IV.
porcine (L407)						K..LS..S...G.NL...N..N...KV...F...R..E...LAQ.LSL.M..IV.
sheep						K..VLNA.....NL.....N..ANV...F...QH.....L.E.LSL...I..
bear, black						K..VL.....N.R.....N.L..F...Q.....L.L.L.L.L...I.C
cat						M..K..L.....R.....R...L...N..MQD.....LYL.L.L...I.C
guinea pig						VLSS.S..R..E.....N.....L.A...F...CD..VK.L.SSI.MT.V.I.C
horse						K.VR.A.....N.....H.T.L...L...Q...T...L.S.L.L...C
hamster						K..LSS...R..N.....KA.S...F.L...EE...L.E.I.NSI..I.C
mouse, house						K.VFS...S..R.DQKLEKM.CQEAN.LCDLI.TYDGESRDLSTLIFKSVINICT
mouse, white-foot						K..LSS...R...R..T....A.S...L.L...EE.V.L.Q.I.MS...I.C
rat, brown						H...K.VFS..S....QKLEKL.CQEAK.LCDM...HDKESIDLSTPIFMSVTNICA

	190	200	210	220	230	240
human	LICFNT	SYKNGD	PELNVI	QNYNEGI	IDNLSK	DSLVDL
baboon, cape						VLPWLK
baboon, yellow						IFPNKT
bison						LEKLSH
bovine						VKIRN
chimpanzee						I.....KIVH.....S.G.E.....F.....V.....R...T..
goat, angora (ACS-)						F...F.F..E..A.KA..V.D..LEV...EV.L.IF.V...S.AM..M.GY.QT..
goat, angora (ACS+)						F...F.F..E..A.KA..V.D..LEV...EV.L.IF.V...S.AM..M.GC.QT..
macaque, crab-eating						I.....KIVH.....S.G.E.....F.....V.....R...T..
macaque, rhesus						I.....KIVH.....S.G.E.....F.....V.....R...T..
porcine (H407)						F...F.F.K...A.QA.V.F.D..L.AVG.EI.Y.MF.GIR.L.SQ...NM.QC.RM..
porcine (L407)						F...F.F.K...A.QA.V.F.D..L.AVG.EI.Y.MF.GIR.L.SQ...NM.QC.RM..
sheep						F...F.F..E..A.KA..V.D..LEV...EV.L.IF.A...S.AM..M.GC.ET..
bear, black						S.....A.KI.K.....L.S.GT.NMA.IF.G.....V...NC..M.D
cat						S.F.....A.KI.....LKT.G..N...IF.V.....M.NY..N.D
guinea pig						M...SV...E..M..VT.RRFTT.FVNS..D.N...IF.....MIRKYTE..G
horse						W...S..MK...A.ETM...HK..LET.E..NV..IF.A...S...MRHC.N..
hamster						I...GI..E.R..I.AT.KSFT...LNS.GN.H...IF...T...VDMI.KN...D
mouse, house						ICFNISFENKDPILTTIQTFTFEGIVDVLGHS.LVDIFPWLKIFPNKNLEMIKEHTKIREK
mouse, white-foot						A...S...E.R..I.TT..TFT...LNC.DNEN...IF...T...I.R...V.D
rat, brown						ICFNISYE..DPKLTAKTFTFEGIVDATGDRNLVDIFPWLTFPNKGLEVIKGYAKVRNE

	250	260	270	280	290	300
human	DLLNKI	LENYKE	KFRSDS	ITNMLD	TLMQAK	MNSDNG
baboon, cape						NAGPDQ
baboon, yellow						DSELSN
bison						HILTTI
bovine						GDIFF
chimpanzee						T..F.....V.....
goat, angora (ACS-)						T..F.....V.....
goat, angora (ACS+)						E...E...KCQ.N.S.....L.HI.I...V.A..N.....K...SR.M.A.....
macaque, crab-eating						E...E...KCQ.N.S.....L.HI.I...V.A..N.....K...NR.M.A.....
macaque, rhesus						T..F.....H.....V.....
porcine (H407)						E..RE...R..NYSRN...L..IMI...T.AESNTG...HNLK...R.M.A.VA...
porcine (L407)						E..RE...R..NYSRN...L..IMI...T.AESNTG...HNLK...R.M.A.VA...
sheep						E..SE...KCQ.N.T.....L.HI...V.A..N.T..E...K...NR.M.A..A...
bear, black						E...E...KH..N.S...SL..V.I..R...G.N..AS.R.AK...K.....
cat						E..RE...KH..N.SN.....V.I..R...NG.AS...K...K.....
guinea pig						AM.S...KEC.....VS.LI.L.I...V.EN.N.SSL...N.F..K...L...
horse						E..S...F.KH..N.N...S...L.I...K...N.T...K...K...A...
hamster						EV.SG...KC...N...SSLM.L.I...T.A..N.TSEG.G.NAF...M...A..A...
mouse, house						T.VEMFEKCKEKFNESLSLTDLIQAQMNAEN.NTGEGQDP.VFSDKHILV.VGDIFG
mouse, white-foot						EV.IDM..KC...NN...S.LT.I.I...A..N.TTDH.G.FSD.HILSTVGDFFGA
rat, brown						V.TGIFEKCREKFDQSISLSLTDLIQAQMNSDN.NSCEGRDPPVFSDRHILA.VGDIFG

	310	320	330	340	350	360
human	GAGVETTTSVVKWTLAFLLHNPQVKKKLYEEIDQNVGFSRPTPTISDRNRLLLLLEATIREV					
baboon, cape	.....IV.....G.....					
baboon, yellow	.....IV.....					
bison	.....I..IV..Y..H..SL..RIQDD...II..N...A.....V.....					
bovine	.....I..IV..Y..H..SL..RIQDD...II..N.....V.....					
chimpanzee	.....					
goat, angora (ACS-)	.....I..IV..Y..H..SL..RIQDS...I..N.....C.V.....					
goat, angora (ACS+)	.....I..IV..Y..H..SL..RIQDS...I..N.....C.V.....					
macaque, crab-eating	.....IV.....					
macaque, rhesus	.....IV.....					
porcine (H407)	.....SA.....IV.....Y..LLR..IQDA...I..N.A.S.....Q.V.....					
porcine (I407)	.....SA.....IV.....Y..LLR..IQDA...I..N.A.S.....Q.V.....					
sheep	.....I..IV..Y..H..SL..RIQDS...I..N.....V.....					
bear, black	.....V.....LQ..IQ.....G..NM...Q.IW.....					
cat	.....R..V.....H..LY..Q.....I..G...M...Q.I.....					
guinea pig	.....SS..L.VI.....D..L..IQ.....H.....F...H..M.....					
horse	.....IV.....D..L..IQ.....L.....					
hamster	...I...A..LS.II.....E...IQK...I.....FN..H..M.....					
mouse, house	AGIET.SSVLNWILAFLVHNPVVKR.IQK.IDQYVGF SRTPSFNDRTHL.M.EATIREVL					
mouse, white-foot	.IETT.SVLCWIVAFLLHNPEVKK.IQKEIDQNVGFSRTP.FNDRNHLLM.EATIREVLR					
rat, brown	AGIET...VLKWLAFLVHNPVVK...IQK.IDQYVGF SRTPPTFNDRSHL.M.EATIREVL					

	370	380	390	400	410	420
human	LRLRPVAPMLIPHKANVDS SIGEFAVDKGTQTEVIINLWALHNEKEWHQPDQFMPERFLNP					
baboon, cape	..I.....H.....					
baboon, yellow	..I.....H.....					
bison	..I....T....VI....DLTI...D.VV....S...QH..L....D.					
bovine	..I....T....VI....DLTI...D.VV....S...QH..L....D.					
chimpanzee	.....Q.....					
goat, angora (ACS-)	..I.....II....DLTI...D.VV.....Q...L....D.					
goat, angora (ACS+)	..I.....II....DLTI...D.VV.....Q...L....D.					
macaque, crab-eating	..I.....H.....					
macaque, rhesus	..I.....H.....					
porcine (H407)	..F...S.T...R.II.....TI..D.D.VV.....R..L....D.					
porcine (I407)	..F...S.T...R.II.....TI..D.D.VV.....LR..L....D.					
sheep	..I....T....II....DLTI...D.VV.....Q...L....D.					
bear, black	..I....T....I.....I...S.....R.....D.					
cat	..I....T....IM.....N.....YR.....D.					
guinea pig	..I....I....T.....I..D.N.LV.....Q..DR.....D.					
horse	..I.....L.....D..N.....R.....					
hamster	..I.....R..S.M...SIP.F.P.....S...D...R.....D.					
mouse, house	RIRPVAPLLIPHKANIDS.IGEFAI PKDTHVI.NLWALH.DKNEWDPDRFMPERFLDPT					
mouse, white-foot	I.PVAPMLIPHKANIDSSIGEFTIPK.THVVINLWALHNEK.WDQPDRFMPERFLDPTG					
rat, brown	RIRPVAPMLIPHKANVDS.IGEFVTPK DTHV.VNLWALH.DENEWDQPDQFMPERFLDPT					

	430	440	450	460	470	480
human	AGTQLISPSVSYL PFGAGPRSCIGEILARQELFLIMAWLLQRFDLEVPD DQGLPSLEGIP					
baboon, cape	.....L.....N.....					
baboon, yellow	.....L.....N.....					
bison	T.....L.....V..M.....F.SR....N..I...K....HA					
bovine	T.....L.....V..M.....F.SR....N..I...K....HA					
chimpanzee	.....					
goat, angora (ACS-)	T.....L.....V..M.....F.SR....N..I...K....N.					
goat, angora (ACS+)	T.....L.....V..M.....F.SR....N..I...K....N.					
macaque, crab-eating	.....L.....N.....					
macaque, rhesus	.....L.....N.....					
porcine (H407)	T.....L.....V..M.....FT.G.....L.....C.V.N.					
porcine (I407)	T.....L.....V..M.....FT.G.....L.....C.V.N.					
sheep	T.....L.....V..M.....F.SR....Y..I...K....N.					
bear, black	TKS.....L.....L.....F.....M.....N.					
cat	TRS.....L.....L..S...V..F.....H...N.					
guinea pig	T.S.I.V.S.....V..A...I...T...K....EG.....					
horse	T.S.....L.....L.....FT.....N.....H.					
hamster	T.SH.T.L.....A.....V.....F..H.....D...E.P.C.K.NA					
mouse, house	GSHLITPTPSYLPFGAGPRSCIGEALARQELFIFMALL.QRFDLVDSD.KQLPCLVGDPK					
mouse, white-foot	SHLITP.L.YLPFGA.PRACIGEV LARQVLF.YVACL LQRFDL.VPDGAPMPC LKGD PKV					
rat, brown	GSHLITPTQSYLPFGAGPRSCIGEALARQELFVFTALL.QRFDLVDSD.KQLPRLEGDPK					

	490	500	510
	..... ..... ..... ..... ..... ..... .		
human	KVVELIDSFKVKIKVRQAWREAQAEGST		
baboon, cape	.....		
baboon, yellow	.....		
bison	SL.LQ.KP.....E.....K.....P		
bovine	SL.LQ.KP.....E.....K.....P		
chimpanzee	.....		
goat, angora (ACS-)	SL.LQ.KP.....E.....K.....S		
goat, angora (ACS+)	SL.LQ.KP.....E.....K.....S		
macaque, crab-eating	.....		
macaque, rhesus	.....		
porcine (H407)	SL.LQ..P.....E.....K..HT....S		
porcine (L407)	SL.LQ..P.....E.....K..HT....S		
sheep	SL.LQ.KP.....E.....K.....S		
bear, black	.L....EP.....S..		
cat	T....AP....V.....		
guinea pig	.I....P....T..P..K.....A		
horse	TA.....N.....		
hamster	N.....P....T....KD...VN.WRP		
mouse, house	V.FLIDPFKVKITVRQAWKDAQVEVST		
mouse, white-foot	VFLLIDPFKV..TVRQAWKDAQ.EVGTWRP		
rat, brown	V.FLIDPFKVKITVRQAWMDAQAEVST		



```

          310          320          330          340          350          360
wt pCYP17A1-VH  GAGVETSASVVKWIVAFLLHYPLLRRKKIQDAIDQNI GFNRAPSI SDRNQLV LLEATIREV
pCYP17A1-LH
pCYP17A1-VL
pCYP17A1-LL
pCYP17A1-LA
Tes. pCYP17A1-LL (1992)
Adr. pCYP17A1-LH (1987)

```

```

          370          380          390          400          410          420
wt pCYP17A1-VH  LRFRPVSPPTLI PHRAIIDS SIGEFTIDKDTDVVVNLWALHHNEKEWHRPDLEMPERFLDP
pCYP17A1-LH
pCYP17A1-VL
pCYP17A1-LL
pCYP17A1-LA
Tes. pCYP17A1-LL (1992)
Adr. pCYP17A1-LH (1987)

```

```

          430          440          450          460          470          480
wt pCYP17A1-VH  TGTQLISPSLSYLPFGAGPRSCVGEMLARQELFLFTAGLLQRFDELPLDDGQLPCLVGNP
pCYP17A1-LH
pCYP17A1-VL
pCYP17A1-LL
pCYP17A1-LA
Tes. pCYP17A1-LL (1992)
Adr. pCYP17A1-LH (1987)

```

```

          490          500          510
wt pCYP17A1-VH  SIVLQIDPFKVKIKERQAWKEAHTEGSTS*LHPG*
pCYP17A1-LH
pCYP17A1-VL
pCYP17A1-LL
pCYP17A1-LA
Tes. pCYP17A1-LL (1992)
Adr. pCYP17A1-LH (1987)

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### ADDENDUM C

Sequence alignment of the 134 amino acids of various mammalian CYB5 enzymes relative to the human form. GenBank accession numbers: human (*Homo sapiens*) [AAA35729.1](#); chimpanzee (*Pan troglodyte*) [JAA42572.1](#); crab-eating macaque (*Macaca fascicularis*) [EHH58972.1](#) and rhesus macaque (*Macaca mulatta*) [AFH31257.1](#) and [AFJ70408.1](#), respectively; marmoset (*Callithrix jacchus*) [JAB11986.1](#); dog (*Canis lupus familiaris*) [ADJ67811.1](#); porcine (*Sus scrofa*) [AAC48779.1](#); shrew (*Tupaia chinensis*) [ELV10907.1](#); rat (*Rattus norvegicus*) [AAB67610.1](#); house mouse (*Mus musculus*) [AAH24341.1](#); angora (*Capra hircus*) [ABQ12619.1](#) and [AEJ84155.1](#), respectively; sheep (*Ovis aries*) [ACV53659.1](#); bovine (*Bos taurus*) [AAI08114.1](#); yak (*Bos mutus*) [ELR50125.1](#); mole (*Heterocephalus glaber*) [EHB06367.1](#) and hamster (*Cricetulus griseus*) [EGW12916.1](#).

	10	20	30	40	50	60	70
human	MAEQSDEAVKYYTLEEEIQKHNSKSTWLI	LHHKVYDLTKFLEEHPGGEEVLR	EQAGGDATENFEDVGHST				
chimpanzee	.....	.....	.....	.....	.....	.....	.....
macaque, crab-eating	.....	.....	.....	.....	.....	T.....	.....
macaque, rhesus1	.....	.....	.....	.....	.....	.....	.....
macaque, rhesus2	.....	.....	.....	.....	.....	.....	.....
marmoset	.....	.....	.....	.....	.....	.....	.....
rabbit	A..	KD.....	K.....	.....	.....	.....	.....
dog	.....	QT.....	.....	.....	.....	.....	.....
porcine	.....	K.....	N.....	.....	.....	.....	.....
shrew	.....	K.....	V.....	.....	.....	.....	.....
rat	.....	KD.....	KD.....	V.....	.....	.....	.....
mouse	G..	KD.....	KD.....	V.....	.....	.....	.....
angoral	E..	SKP.....	.....	Y.....	.....	.....	.....
angora2	E..	SKP.....	.....	Y.....	.....	.....	.....
sheep	E..	SKP.....	.....	Y.....	.....	.....	.....
bovine	E..	SK.....	N.....	Y.....	.....	.....	.....
yak	E..	SK.....	N.....	Y.....	.....	.....	.....
mole	A..	E..QT.....	KNN.....	V.....	Y.....	S.....	.....
hamster	G..	KE.....	KD.....	V.....	.....	.....	.....

	80	90	100	110	120	130	140
human	DAREMSKTFIIGELHPDDRPKLNKPPETLIT	TIDSSSSWWTNWVI PAISAVAVALMYRL	MAED				
chimpanzee	.....	.....	.....	.....	.....	.....	.....
macaque, crab-eating	.....	Y.....	S.....	V.....	F.....	.....	.....
macaque, rhesus1	.....	Y.....	S.....	V.....	F.....	.....	.....
macaque, rhesus2	.....	Y.....	S.....	V.....	.....	.....	.....
marmoset	L..	Y.....	.....	S.....	V.....	G.....	.....
rabbit	L..	.....	S..S..M.....	V..N.....	LI.....	D.....	.....
dog	L..	.....	A..I..S.....	V..N.....	LV.....	HF..T...	.....
porcine	L..	.....	S..IA..S.....	VE..N.....	LV..S...	HF..TS..N	.....
shrew	L..	Y.....	S..IT.....	LE..N.....	L..V..L.....	I..V...	.....
rat	L..	Y.....	S..IA..S.....	VE..N.....	LV.....	.....	.....
mouse	L..	Y.....	S..IA..SD.....	VE..N.....	L.....	.....	.....
angoral	L..	.....	S..IT..S..SI.....	N.....	L.....	LV.....	H..TS..N
angora2	L..	.....	S..IA..S..SI.....	NP.....	L.....	LV.....	H..TS..N
sheep	L..	.....	S..IT..S..SI.....	N.....	L.....	LV.....	H..TS..N
bovine	L..	.....	S..IT..S..SI.....	NP.....	L.....	LF...I..H..TS..N	.....
yak	L..	.....	S..IT..S..SI.....	NP.....	L.....	LF...I..H..TS..N	.....
mole	L..	.....	V.....	A..S..S.....	VE..N.....	G...L...M...F..I...	.....
hamster	L..	Y.....	S..IA..AG..ALRLAANGCLLYFICAAGSY..	WWTNWVI PAISALAVALMYRL	.....	.....	.....