Pichia pastoris: a viable expression system for steroidogenic cytochrome P450 enzymes

by

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been subjected to any university for a degree.

SUMMARY

This study describes:

i. The cloning of the CYP19 gene and construction of the intracellular expression vector pPIC3.5K-CYP19.
ii. The transformation of the yeast, *Pichia pastoris* with the constructed vector.
iii. The expression of $P450_{arom}$ in *Pichia pastoris*.
iv. The determination of enzyme activity and isolation of the protein from the *Pichia pastoris* cells.
v. The expression of P450c17 in *Pichia pastoris*.
vi. The determination of kinetic constants for the conversion of progesterone to 17OH-progesterone and 16OH-progesterone by P450c17.

OPSOMMING

Hierdie studie beskryf:

i. Die klonering van die CYP19 geen en die konstruksie van die intrasellulêre uitdrukkingsplasmied, pPIC3.5K-CYP19.
ii. Die transformasie van die gis, *Pichia pastoris,* met die gekonstrueerde plasmied.
iii. Die uitdrukking van aromatase in *Pichia pastoris*.
iv. Die bepaling van ensiemaktiwiteit en die isolering van die proteïen vanuit *Pichia pastoris*.
v. Die uitdrukking van P450c17 in *Pichia pastoris*.
vi. Die bepaling van kinetiese konstantes vir die omsetting van progesteroon na 17OH-progesteroon en 16OH-progesteroon deur P450c17.

Met liefde opgedra aan

my man

&

ouers

It is by logic that we prove, but it is by intuition that we discover.

(Henri Poincaré)

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Aan God die drie-enige, kom toe die lof, die eer en die heerlikheid, tot in alle ewigheid, Amen.

Abbreviations

17βHSD	17β-hydroxy-Δ^5-steroiddehydrogenase-isomerase
3βHSD	3β-hydroxy-Δ^5-steroiddehydrogenase-isomerase
ACTH	Adrenocorticotropin
ADX	Adrenodoxin
ADXR	Adrenodoxin reductase
AOX1	Alcohol oxidase gene
Aox1p	Alcohol oxidase
AR-1	Activator protein 1
AR-2	Activator protein 2
BMGY	Buffered glycerol complex medium
BMMY	Buffered methanol complex medium
bp	Base pair
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CIAP	Calf intestinal phosphatase
CO	Carbon monoxide
COX1	Cyclooxygenase 1
COX2	Cyclooxygenase 2
cpm	Counts per million
CRE	*cis*-acting cAMP regulatory elements
CYP17	Cytochrome P450 17α-hydroxylase gene
CYP19	Cytochrome P450 19α-hydroxylase gene

Da	Dalton
DHEA	Dehydroepiandrosterone
E1	Estrone
E2	Estradiol
EC	Endometrium cancer
EDTA	Ethylenediaminetetra-acetic acid disodium salt
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FAD	Flavine adenine dinucleotide
FMN	Flavine mononucleotide
HPLC	High Performance Liquid Chromatography
IMAC	Immobilized metal affinity chromatography
kb	Kilo bases
kDa	kilo Dalton
MD	Minimal dextrose
MM	Minimal methanol
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
ODEC	3-oxodecalin-4-ene-10-carboxaldehyde
$P450_{arom}$	Product of the CYP19 gene
P450c17	Product of the CYP17 gene
P450c21	Product of the CYP21 gene
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PMSF	Phenyl methyl-sulfonyl fluoride
RT-PCR	Reverse transcriptase polymerase chain reaction

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
UV	Ultra violet
YPD	Yeast extract peptone dextrose medium

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CHAPTER ONE

1.1 Introduction

Cytochrome P450 enzymes are a family of heme-containing enzymes, defined by their characteristic enzymatic function as monooxygenases [1,2]. They are so-called because of the characteristic band displayed at 450nm, by the ferrous (Fe^{2+}) carbon monoxide complex in the proto porphyrin IX heme nucleus. Cytochromes P450 play a large role in the detoxification of xenobiotics in the body (exogenous substrate metabolism). In addition to this, the cytochrome P450 enzymes are also responsible for the metabolism of steroids, vitamins, fatty acids and eicosanoids (endogenous substrate metabolism). These enzymes are found in all five of the biological kingdoms, with the exception of certain primitive species of bacteria [2,3]. Although the cytochromes P450 are present in most tissues in mammalian species, they are predominantly found in the endoplasmic reticulum (ER) membrane of hepatocytes in the liver. In addition to this major expression site for cytochromes P450, there are also various other forms expressed in other tissues and organs, such as the kidneys, brain, breast, nasal epithelium, prostate, gonads, skin, spleen, lung, gastro-intestinal tract and pancreas [2,4]. For the conversion of cholesterol to steroid hormones, a sequence of cytochrome P450-dependent monooxygenation reactions, together with Δ-5 to Δ-4 isomerization coupled to NAD-dependent oxidation of the 3β-hydroxyl function, is required. There are three microsomal cytochrome P450 enzymes that partake in steroidogenesis, namely P450c21 (21-hydroxylase), P450c17 (cytochrome P450 17α-hydroxylase/17,20-lyase) and aromatase (product of the CYP19 gene), the latter two will form the focus of this study [2,5].

P450c17 is expressed in the adrenals, ovaries and testis and catalyses two different reactions. The first is the relative simple 17α-hydroxylation of pregnenolone and progesterone to yield 17α-hydroxypregnenolone (17OHpreg) and 17α-hydroxyprogesterone (17OHprog), respectively. The second reaction is a 17, 20 oxygenase-dependent cleavage, also known as a lyase reaction, to yield

dehydroepiandrosterone (DHEA) from 17OHpreg and adrostenedione from 17OHprog [2,5,7].

Aromatase displays tissue-specific regulation and is expressed in the ovaries, placenta, brain and adipose tissues. This enzyme catalyses three sequential oxygenation reactions that convert C19 androgens to aromatic C18 estrogens. This three step oxidative process includes both aromatase and the flavoprotein NADPH-cytochrome P450 reductase, and requires three equivalents of molecular oxygen and NADPH per estrogen molecule formed [2,6,8,9]. The increased incidence of both breast cancer and endometrial cancer (EC) can be correlated to estrogen biosynthesis by adipose tissue that increases with body weight and age [10]. Together, these two microsomal P450 enzymes, P450c17 and aromatase, play an integral role in steroidogenesis, more specifically in the production of sex steroids. Androgens are produced *via* DHEA, one of the products of the lyase activity of P450c17. Androgens are converted by aromatase to estrogens. Reduced P450c17 activity or a non-functional enzyme leads to a decrease in sex steroids production in males and can result in ambiguous genital formation, while in females, puberty may never be reached. Breast cancer as well as EC is believed to develop and progress as a result of abnormal expression of aromatase [11,12].

Chapter two of this thesis describes the structure, spectral properties and general catalytic mechanisms of cytochrome P450-dependent enzymes. This chapter also reviews the role of cytochromes P450 in steroidogenesis, the location of these enzymes and the electron donor systems. In chapter three, aromatase is discussed in depth in terms of physical properties, role in steroidogenesis, membrane topology, localization and its role in female cancers.

For the expression of aromatase in the yeast *Pichia pastoris*, it was necessary to construct an expression vector containing the *CYP19* gene. The cloning of *CYP19* gene into *Escherichia coli* and two different approaches to transform *P. pastoris* with the constructed expression vector, are discussed in Chapter four. This Chapter also discusses the expression of aromatase in *P. pastoris* as well as the characterization, isolation and purification of the enzyme from isolated yeast microsomes. The results obtained from the cloning, electroporation, expression and purification are also presented and discussed.

In Chapter five, an overview is given of P450c17 in terms of physical properties, zonal distribution, membrane topology and its role in adrenal steroidogenesis and cancer. Chapter six discusses the growth conditions of the *P. pastoris* cells, transformed with P450c17, as well as the kinetic studies that were conducted with this enzyme. This Chapter also presents the results obtained from the work that was done on the P450c17 enzyme, followed by a discussion. In the conclusion, a summary of the data obtained from this study is given and presents an overview of the main points concluded from this work.

CHAPTER TWO

Cytochromes P450

2.1 Introduction

In 1955, G. R. Williams and M. Klingenberg independently noticed a pigment with an atypical carbon monoxide-binding spectrum in rat liver microsomes [1]. This pigment, when reduced, had an absorption maximum at 450nm. Cytochrome b_5 has an absorption maximum at 420nm when reduced and was the only known hemoprotein in rat liver microsomes at that time. In 1962, Tsuneo Omura and Ryo Sato [1] published their findings and proposed the name "P450" for this "new" hemoprotein, which means "a pigment with an absorption at 450nm". When microsomes were treated with a detergent or a phospholipase preparation, the active cytochrome P450 was converted to cytochrome P420 (P420), the inactive form of the enzyme. It was established that P420 also had hemoprotein properties and this confirmed that the membrane bound cytochrome P450 should also be a hemoprotein [1,2]. A unique feature of cytochrome P450 hemoproteins is a thiolate in the active centre, which is ligated to the iron atom of protoporphyrin IX [13]. Since the discovery in liver microsomes, many other forms of cytochrome P450-dependent enzymes have been found in both the ER and the mitochondria. These enzymes are also called the monooxygenases and they activate molecular oxygen with the resultant oxidation of the substrate. A single oxygen atom is incorporated into an organic substrate to produce a molecule of water and a monooxygenated metabolite. Many different reactions are catalysed by the cytochrome P450 monooxygenases, such as the hydroxylation of carbons, the oxygenation of nitrogen, sulfur, halogens and phosphorus as well as the epoxidation of double bonds. Substrates for cytochromes P450 are endogenous as well as exogenous and include fatty acids, lipids, vitamins, steroids, carcinogens and xenobiotics [1-4,7,13].

2.2 The general structure of cytochromes P450

The molecular mass of cytochromes P450 varies between 44 and 60 kilo Dalton (kDa), as determined by SDS-gel electrophoresis [14-18]. The enzyme comprises a single polypeptide chain and typically about 45 to 55 percent of the amino acid residues are non-polar and the amino terminal residues are effectively free. As previously mentioned, cytochromes P450 have an iron protoporphyrin IX as a prosthetic group and is part of the b-type hemoproteins [2,14-18]. This prosthetic group is located in a large hydrophobic cleft. The heme group is loosely bound by a combination of hydrophobic interactions, Coulombic attractions and covalent bonds. A cysteine thiolate anion is the 5^{th} axial ligand of the hemoprotein. The presence of this 5^{th} ligand ensures the unique ability of the enzyme to undergo autoxidation. The hydrophobic environment of the active site is crucial to the ionization of the –SH group. If this environment is disturbed, the enzyme is converted to its inactive form, cytochrome P420, as the thiolate anion cannot serve as the 5^{th} ligand anymore. The 6^{th} ligand is bound weakly to the heme group and dissociates when iron reduction takes place or is easily replaced. This ligand is in a highly conserved region of the protein and plays a key role in the formation of the oxygen binding site where it acts as a binding site for molecular oxygen upon reduction of the heme group [13,14,16,17]. Fig. 2.2.1 is a schematic representation of the active site of cytochrome P450 enzymes. The S represents a sulfur ligand [21].

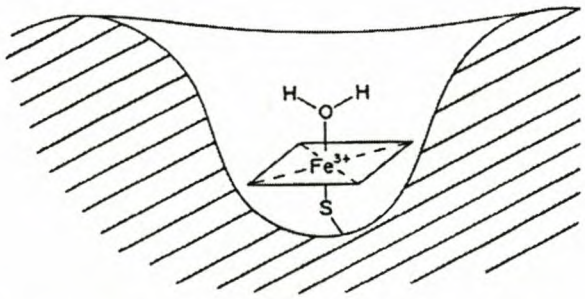


Fig. 2.2.1. Model of the active site of cytochrome P450-dependent enzymes.

2.3 Spectral properties

Upon replacement of the 6th ligand, the spin state of the Fe^{3+} in the protoporphyrin IX prosthetic group of cytochrome P450 changes from a low spin, hexa-coordinated shape, to a high spin, penta-coordinated shape as shown in Fig. 2.3.1.

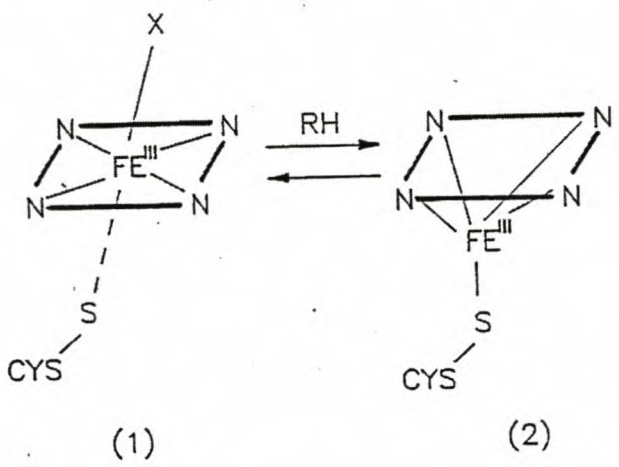


Fig. 2.3.1. Position of the low spin (1) and high spin Fe^{3+} in the ring [13].

During catalysis the 6th ligand is replaced by the substrate which leads to the uptake of electrons, allowing for the binding of oxygen to the complex. When binding of the substrate occurs the iron is displaced from the plane of the pyrole ring towards the thiolate sulphur [13,19]. This change can be determined spectrophotometrically. Important information about the active site, as well as the mode of action of cytochrome P450 dependent enzymes, has been gleaned from their characteristic difference spectra [4,13]. There are three different types of substrate binding, namely Type I, Type II and reverse type I, also known as modified Type II. These types relate to the heme iron spin-state modulation together with ligation at the distal heme face. Type I is a shift in the spin equilibrium towards high spin. The shift in the Soret band absorption maximum is at 420nm with an associated increase of the 390nm absorption in ferric cytochrome P450 UV spectrum. Type II binding refers to a decrease in UV absorption at 390 – 405nm with an increase at ~ 425 – 435nm. This is a shift towards low spin ferric state. It indicates a direct heme iron ligation by substrate cytochrome P450 inhibition. Reverse Type I or

modified Type II is essentially a 'mirror image' of Type I. It is thought that iron heme ligation does not occur in this instance [2,4,16].

2.4 General catalytic cycle of cytochromes P450

The cytochrome P450 catalytic cycle essentially involves the combination of oxygen with the organic substrate (RH) to produce a molecule of water and a mono-oxygenated metabolite (ROH). Most of the reactions begin with the transfer of electrons in two consecutive steps once the substrate has bound. The first electron is transferred from NAD(P)H to either NADPH-cytochrome P450 reductase in the microsomal system, or to a ferredoxin reductase and a nonheme iron protein in the mitochondrial and bacterial systems. The second electron is transferred to cytochrome P450 leading to the reductive activation of molecular oxygen followed by the insertion of one oxygen atom into the substrate to form a mono-oxygenated metabolite while the other oxygen atom is incorporated in a molecule of water [4,16,20,21]. The general reaction equation of the cytochrome P450 catalytic cycle can be written as follows:

$$RH + O_2 + NAD(P)H + H^+ \longrightarrow ROH + H_2O + NAD(P)^+$$

Substrates, for the vast variety of cytochrome P450 enzymes are mostly lipophilic such as the fatty acids, eicosanoids, steroids, lipid hydroperoxides and retinoids. Some of the substrates are highly hydrophilic despite earlier beliefs that they were mostly hydrophobic. Some other substrates for cytochromes P450 include the xenobiotics, antioxidants, pesticides, herbicides, alcohols, solvents and other foreign substances. With so many different substrates, it comes as no surprise that this unique group of enzymes is capable of many different reactions including hydroxylation, peroxygenation, dehalogenation, epoxidation, desulfuration as well as reduction and it is peculiar that, more than forty years after they were first discovered, these enzyme are still collectively known as the cytochromes P450. This name, first used to describe a red pigment with a reduced CO difference spectrum at 450nm, seems unsuitable as it does not describe the biological functions of the enzymes at all. Even the term cytochrome in the name is an anomaly, since cytochrome P450-dependent enzymes do not act purely as electron

carriers as cytochromes normally do. In the light of this controversy, it has been suggested that the name should be changed to diversozymes [1-4,20,22]. Some of the transformation reactions carried out by the cytochromes P450 is vital for life, e.g. the conversion of cholesterol to the corticoid and sex hormones. Other reactions, like the detoxification of xenobiotics lead to more polar compounds that can be more easily excreted. The catalytic cycle of a typical cytochrome P450 catalysed reaction can be summarized in four steps:

a. Substrate binding with displacement of the sixth ligand inducing a shift in the absorbance maximum, spin state and redox potential of the hemoprotein system.
b. One-electron reduction of the complex to a ferrous state, driven by the increase in redox potential that results from the previous step.
c. Binding of molecular oxygen to give a superoxide complex.
d. A second reduction step leading to an 'activated oxygen species'.

2.4.1 Stages in the catalytic cycle of cytochrome P450-dependent enzymes

Fig. 2.4.1 is a schematic representation of the catalytic cycle of cytochrome-dependent P450 enzymes, while Table 2.4.1 summarizes the different spin states of the heme iron [14].

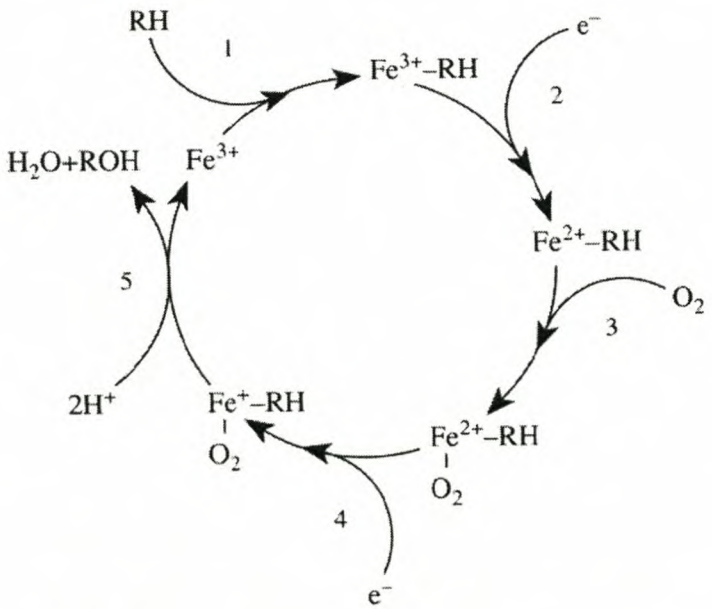


Fig. 2.4.1. Catalytic cycle of cytochrome P450-dependent enzymes. RH represents a substrate and ROH the corresponding product. The heme iron redox state is also shown for each stage [3].

Table 2-1 d-Orbital electron arrangement of the heme iron with the cytochrome P450 substrate-free and substrate-bound [14].

Coordination Valence	Ferric Spin State	Iron d Electrons	Soret Band (nm)	Substrate
6	Low	↑↓↑↓↑	420	Absent
5	High	↑↑↑↓↓	390	Bound

The first step in the reaction cycle of cytochrome P450 is substrate binding. Ferric cytochrome P450 combines with the substrate to form an enzyme-substrate complex. This disturbs the spin-state equilibrium and aids the uptake of the first electron. It is believed that the main contributor to the binding energy is the hydrophobic effect. Substrate binding causes the coordination sphere to change from hexacoordinated to pentacoordinated, as mentioned earlier. This also results in changes in the *d*-orbitals and the electron pairing pattern shifts from ferric low spin (S = ½) to ferric high spin (S = 5/2). The high spin iron moves out of the plane of the pyrole ring towards the thiolate sulfur, the reason for this being that the high spin iron has a larger ionic radius and can no longer fit in the central cavity of the porphyrin IX nucleus [4,16,21].

The second step in the catalytic cycle is the one-electron reduction of substrate-bound cytochrome P450. This reducing equivalent can be provided by either NADH or NADPH, depending on the type of system i.e. mitochondrial or microsomal electron transport system. Once the redox partner provides an electron to the enzyme-substrate complex, the ferric iron of the hemoprotein is reduced to the ferrous form while remaining high spin. This switch from ferric to ferrous state, allows molecular oxygen to bind strongly to cytochrome P450 through heme ligation [4,16,21,23].

Dioxygen binding is the third step in the catalytic cycle. It involves the interaction between ferrous substrate-bound cytochrome P450 and dioxygen. This results in an oxyferro complex of cytochrome P450 also known as oxycytochrome P450 and is in effect the first step of oxygen activation [14,15,16,18].

In the 4th step of the cycle, the second electron transfer activates O_2 to O_2^{2-}. At the same time an internal rearrangement of the electrons takes place to restore the iron to its ferric (Fe^{3+}) state. This step initiates dioxygen bond cleavage to yield the catalytically active species [7,13,16,20].

The 5th step involves dioxygen bond cleavage, which consequently leads to one oxygen atom being inserted into the substrate while the other oxygen atom is reduced to a water molecule. After these steps are completed, the hydroxylated product can dissociate from the cytochrome P450 enzyme active site. This completes the catalytic cycle and the free oxidized form of the cytochrome P450 can again enter into the next cycle [4,16,21,24].

2.5 The electron donor systems

2.5.1 The mitochondrial electron transport system

Mitochondrial and most bacterial cytochrome P450-dependent systems consist of three components: ferredoxin (an iron-sulfur protein), NAD(P)H-ferredoxin reductase and cytochrome P450. Adrenodoxin (ADX) is an adrenal ferredoxin and is found in the matrix and on the inner membrane of the mitochondria. ADX transfers electrons from NAD(P)H-adrenodoxin reductase (ADXR) to the adrenal mitochondrial cytochromes P450, $P450_{scc}$ (cytochrome P450 side chain cleavage) and $P450_{11\beta}$. The ADX iron-sulfur centre consists of two iron atoms that are tetrahedrally coordinated by four cysteine residues and two sulfur atoms. Only a single electron can be introduced into the active centre of oxidized ADX. ADXR is found on the matrix side of the inner mitochondrial membrane and is highly specific for NADPH. It contains only one molecule of FAD. The flow of electrons from the reduced pyridine nucleotide to cytochrome P450 can be presented as follows:

$$\text{NADPH} \rightarrow \underset{(\text{FAD})}{\text{ADXR}} \rightarrow \text{ADX} \rightarrow \text{P450}$$

Studies by Lambeth *et al.* [25,26] provided the basic information that describes the steroidogenic electron transport in adrenal mitochondria. NADPH donates an electron to ADXR which in turn transfers it to the oxidized form of ADX. ADX first forms a complex with NADPH-reduced ADXR from which it accepts an electron. ADX dissociates from ADXR and acts as a one electron shuttle to reassociate with the membrane bound cytochrome P450 to donate the electron to the hemoprotein. After the electron has been donated to the hemoprotein, the reduced ADX is ready to receive

another electron from ADXR [2,25,26,27]. Fig. 2.5.1 shows a schematic representation of the mitochondrial electron transport system [27].

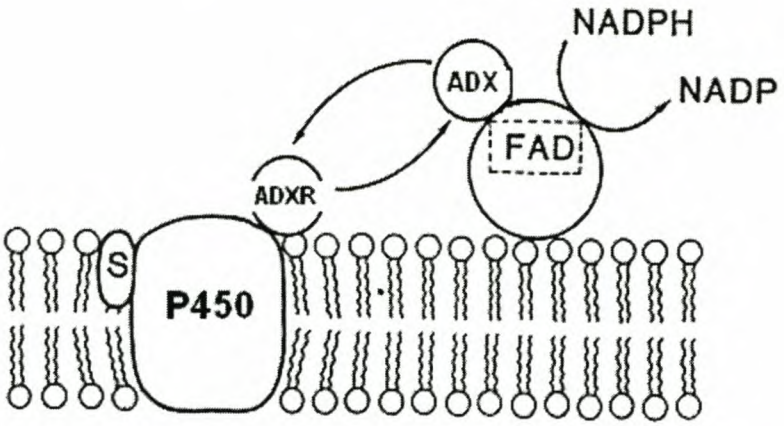
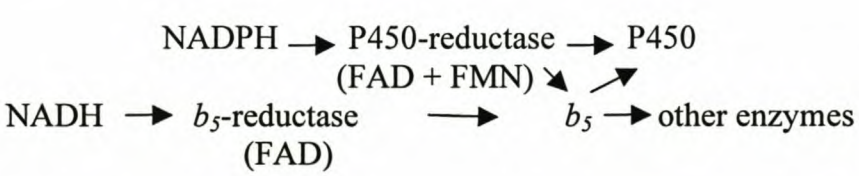


Fig. 2.5.1. Schematic representation of the steroidogenic mitochondrial electron transport system [26].

2.5.2 The microsomal electron transport system

The eukaryotic microsomal electron transport system consists of two components: NADPH-cytochrome P450 reductase and cytochrome P450, which are both membrane bound. There is not, as is the case in the mitochondria, an iron-sulfur protein that acts as an electron shuttle. The flow of electrons from the reduced pyridine nucleotides to cytochrome P450 in the microsomal transport system can be presented as follows:



The NADPH-cytochrome P450 reductase contains one molecule of FMN and one molecule of FAD. In the mitochondrial system, only one FAD molecule is found in ADXR. Two reducing equivalents are supplied from NADPH by the NADPH-cytochrome P450 reductase to cytochrome P450. Studies by Vermilion and Coon [28] yielded vital information on the functioning of the microsomal electron transport system. It was hypothesized that the FAD molecule is a low-potential flavin, which serves as an

electron acceptor from NADPH. The high-potential flavin, FMN, acts as an electron carrier in the transfer process from NADPH to cytochrome P450. FAD can accept two reducing equivalents from NADPH, while FMN serves as the one electron shuttle between NADPH and cytochrome P450 [2,27,28]. In liver microsomes there are two types of electron transport systems. The one system is as described above, while the other system has NADH-cytochrome b_5 reductase as a flavoprotein reductase. The NADH-cytochrome b_5 reductase accepts two electrons from NADH and subsequently transfers it to the one-electron acceptor cytochrome b_5, in an electron transport pathway terminating in either cytochrome P450 in the ER or fatty acyl CoA desaturase. NADH-cytochrome b_5 reductase, a membrane-bound amphipathic flavoprotein contains one molecule of FAD [29]. Fig. 2.5.2 is a schematic representation of the microsomal electron transport system [27].

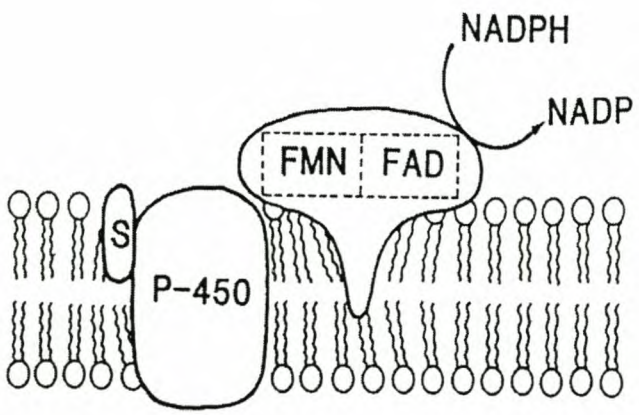


Fig. 2.5.2. Schematic representation of the steroidogenic microsomal electron transport system [26].

2.6 The role of cytochromes P450 in steroidogenesis

Cholesterol is the precursor of all steroid hormones. These steroid hormones are biosynthesized through a sequence of cytochrome P450-dependent monooxygenation reactions. The steroidogenic cytochrome P450–dependent enzymes, responsible for steroid biosynthesis, are found in the adrenal cortex, testis, ovary, placenta, brain and adipose tissues. There are four major steroid hormones that differ from cholesterol in their A and B rings, namely cortisol, aldosterone, androstenedione and estradiol. The

differences in the rings of the first three hormones are brought about by a prominent microsomal enzyme, 3β-hydroxysteroid-Δ^5-isomerase-dehydrogenase (3βHSD), and not by the cytochromes P450. The first step in the biosynthesis of steroids, the conversion of cholesterol to pregnenolone in the mitochondria, is catalysed by the cytochrome P450-dependent cholesterol side chain cleavage enzyme, $P450_{scc}$. The formation of pregnenolone involves three successive reactions, i.e. two hydroxylase reactions at positions 22 and 20 of cholesterol, which is followed by the cleavage of the vicinal diol to pregnenolone and aldehyde. Pregnenolone moves from the mitochondria into the microsomes for the subsequent steps in steroidogenesis. The intermediates of adrenal steroidogenesis must return to the mitochondria for either the production of aldosterone, a mineralocorticoid in the *zona glomerulosa,* or the formation of glucocorticoids in the *zona fasciculata*. During the microsomal steps, three cytochromes P450 are involved. P450c17 can catalyse either a 17α-hydroxylation of progesterone or pregnenolone or it can catalyse a lyase reaction, which is the 17,20 oxygenase dependent cleavage of either 17OHpreg or 17OH-prog to yield DHEA or androstenedione, respectively. Progesterone or 17OHprog also undergoes a 21-hydroxylation reaction, which is catalysed by P450c21, expressed only in the adrenal cortex. The third microsomal cytochrome P450 involved in steroidogenesis is aromatase. This enzyme, like $P450_{scc}$, catalyzes three sequential oxygenation reactions to convert C19 androgens to aromatic C18 estrogens [2,5,6,8,9,14,15,30]. Fig. 2.6.1 shows the steroid hormone biosynthesis pathway.

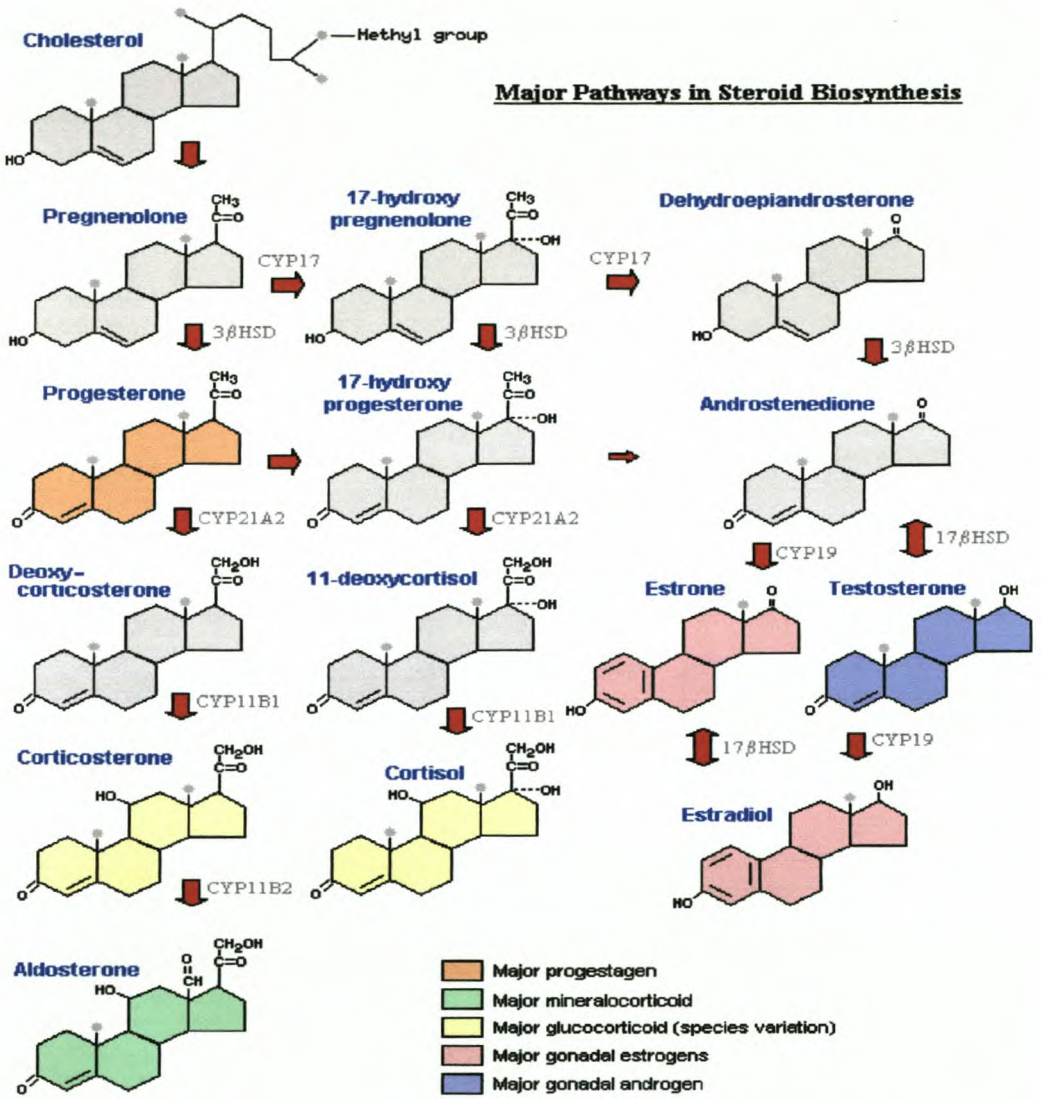


Fig. 2.6.1. Major pathways in steroid hormone biosynthesis

2.7 Summary

The cytochromes P450 are a large, diverse group of unique enzymes that not only catalyse the conversion of endogenous substrates, but also exogenous substrates. These reactions are vital for the sustained health and development of the organism in the production of steroid hormones. Cytochrome P450-dependent reactions are also involved in detoxification and toxic substances are converted to soluble, more readily excreted products. All cytochrome P450-dependent enzymes have the same general structure, an

iron protoporphyrin IX ring as prosthetic group that is located in a large hydrophobic cleft on the surface of the cytochrome P450 protein. The 5th axial ligand of the hemoprotein is a cysteine thiolate anion and this ligand is responsible for some of the unique chemical and physical properties of this enzyme class.

There are two major groups of cytochromes P450, depending on their location. These two groups are the mitochondrial and microsomal cytochromes P450. The most unique characteristics of these enzymes are their spectral properties. The substrate and specific function of the cytochrome P450 can lead to slight changes in the general catalytic cycle that is applicable to all cytochromes P450. The catalytic cycle can be summarized in four steps: substrate binding, a single electron reduction of the substrate-enzyme complex, binding of molecular oxygen and a second reduction step that leads to product formation. The mitochondrial cytochromes P450 utilize ADXR (a flavoprotein with one molecule FAD) and ADX (an iron-sulfur protein) to transfer electrons from NADPH to cytochrome P450. The microsomal cytochromes P450 use only a flavoprotein, with both FAD and FMN as an electron carrier.

During steroidogenesis, cholesterol is the precursor of all steroids and is converted to pregnenolone by mitochondrial $P450_{scc}$. P450c17 has a dual activity, either 17α-hydroxylations or a 17, 20 oxygenase dependent reaction. P450c21 catalyses 21-hydroxylation reactions in the microsomes with either progesterone or 17OHprog as substrates. The microsomal aromatase is responsible for the conversion of androgens to aromatic estrogens.

It is clear that cytochromes P450 play an essential role in biological processes in humans for maintaining the health and wellbeing of the individual. Mutations or malfunctioning of genes encoding cytochromes P450 that are required for endogenous metabolism, may give rise to human diseases such as female cancers, i.e. breast cancer, EC as well as some male cancers. Aromatase and P450c17 have been linked to these disease states and it would thus prove useful to have a heterologous expression system for these proteins, producing sufficient amounts of protein for further studies including kinetic assays,

enzymology, immunohistochemistry and immunocytochemistry. Since there is such a vast majority of cytochromes P450, we are still at the early stages of understanding their many roles in human homeostasis. Therefore the challenge is to better define their role in metabolism and to develop methods for determining their expression levels and activities *in vivo*.

CHAPTER THREE

Cytochrome $P450_{arom}$

Cytochrome $P450_{arom}$ ($P450_{arom}$), also referred to as aromatase, is the product of the *CYP19* gene. *CYP19* is part of the P450 superfamily of genes comprising over 300 members in about 36 gene families. Aromatase displays tissue-specific regulation and is expressed in the male and female gonads, brain, placenta and adipose tissue among others [2,6,8,9,31]. This enzyme is essential for the biosynthesis of estrogens from androgens (Fig. 3.1) and is unique in that it catalyses three sequential hydroxylations to produce an aromatic C18 estrogen. This entire reaction is known as aromatization since it involves the conversion of the Δ^4-3-one A-ring of the androgens to the phenolic A-ring, distinctive of estrogens [2,6,8,9,31].

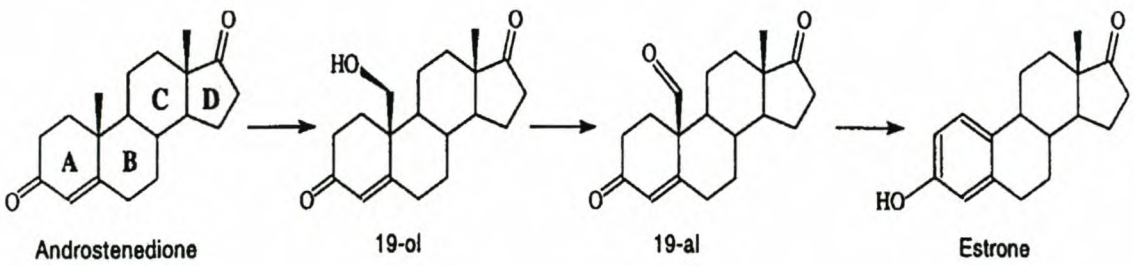


Fig. 3.1. Conversion of androgens to estrogens by aromatase [8].

3.1 The big question: Is aromatase a cytochrome P450?

In 1974, Thompson and Siiteri [32] posed the question whether a P450-dependent enzyme can be responsible for the aromatization of adrostenedione. It was already known that the placental aromatase was a microsomal enzyme. Thompson and Siiteri [32] established that the addition of adrostenedione to placental microsomes, which contained NADPH, led to an increase in the rate of NADPH and oxygen consumption associated with estrogen production. Thorough inhibition studies with KCN led Thompson and

Siiteri [32] to the conclusion that the production of one mole of estrogen required 3 moles of oxygen and 3 moles of NADPH. This stoichiometry suggested that aromatization was brought about by three mixed function oxidation reactions, involving three sequential hydroxylations [32]. Their data also showed that 19-oxoandrostenedione precedes the formation of estrogen, suggesting that the second intermediate must be either the 19-aldehyde or the 19-geminal diol. Subsequent experiments proved the latter [32]. The stoichiometry of aromatization, as well as the nature of the first two hydroxylations that were elucidated, suggested that a single species of cytochrome P450 catalyzed the mixed function oxidations in the process of converting androgens to estrogens. By this time, however, other researchers showed that the aromatization of adrostenedione was insensitive to carbon monoxide (CO) [33,34]. It was believed by some that this evidence was sufficient to prove that a cytochrome P450-dependent enzyme was not involved in the aromatization of C19 steroids [35]. Other reports, however, indicated that CO insensitivity was not the only measure for the determination of cytochrome P450 involvement in this reaction [36]. This dispute encouraged Thompson and Siiteri [32,37] to broaden their investigation into the likely involvement of cytochrome P450 in aromatization as the CO inhibition studies were not sufficient to provide conclusive evidence for the involvement of a cytochrome P450 in the aromatization of C19 steroids. They used different approaches, such as spectrophotometric investigation of the binding of various steroids to placental microsomes, immunochemical techniques and chemical inhibitor studies to establish unambiguously if a cytochrome P450-dependent enzyme was indeed involved in the formation of the sex steroid precursors [37].

The previous observations of the 3 O_2 : 3 NADPH : 1 estrogen stoichiometry for the aromatization of C19 steroids, and the presence of cytochrome P450 in human placental microsomes at the same concentrations found in other steroidogenic tissues, indicated that cytochrome P450 was involved in aromatization [32]. Results from studies with known cytochrome P450 inhibitors were also consistent with the involvement of a cytochrome P450. Thompson and Siiteri [37] showed that there was competitive binding to cytochrome P450 by androstenedione, 19-hydroxyandrostenedione and 19-oxoandrostenedione. These results indicated that all the reactions involved in C19

aromatization were catalysed at the same active site and suggested that a single enzyme complex was responsible for the aromatization of both C18 and C19 steroids [37]. From this ground-breaking work Thompson and Siiteri concluded that a single species of cytochrome P450 was responsible for the aromatization of both C18 and C19 steroids through three consecutive hydroxylations [37]. Their work showed that conclusions could not be drawn on the participation of cytochrome P450 in a given reaction based on CO inhibition studies alone [32,37]. The following mechanism was proposed for the hydroxylations catalyzed by all microsomal cytochrome P450 (Fig. 3.1.1) [37].

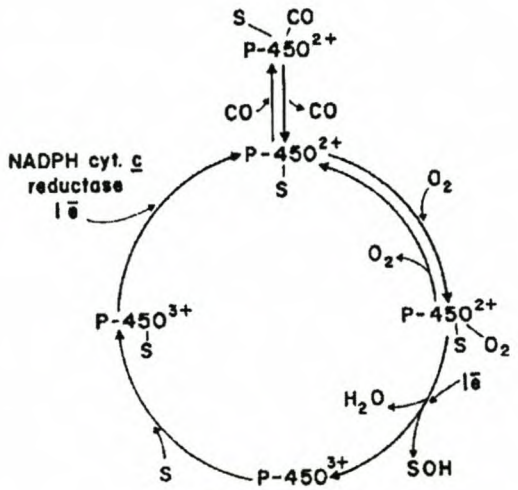


Fig. 3.1.1. Proposed mechanism (as suggested by Thompson and Siiteri) of all microsomal cytochrome P450-catalyzed hydroxylations [37].

By 1985, it was still unclear if aromatase was a cytochrome P450-dependent enzyme and if three sequential hydroxylations took place to produce an estrogen. In a critical and comprehensive review of data available in 1985, Hall [15] again raised the problem that the conversion of adrostenedione to estrone was not inhibited by CO. From the available data Hall [15] proposed that the hydroxylation at C19 was followed by oxidation to a ketone. This step could then be followed by the oxidative elimination of formic acid. However, the reaction mechanism of step II remained unclear. Two possibilities were proposed: the one being a second hydroxylation by monooxygenation whereby the unstable 19-geminal diol would spontaneously collapse to the ketone. The second

possibility was the dehydrogenation of the single hydroxyl group in 19-hydroxyandrostenedione that would lead to the formation of 19-ketoandrostendione. Hall [15] suggested that the use of enzymology and photochemistry would be the only methods to prove the involvement of cytochrome P450 in any reaction beyond any doubt. Since various research groups encountered problems with these methodologies, Hall [15] concluded that it was still impossible to say with certainty that a single cytochrome P450 enzyme was involved in aromatization.

Corbin *et al.* [38] conducted studies on the full-length cDNA encoding human aromatase. They derived the amino acid sequence of human aromatase from the open reading frame and compared it with that of other forms of cytochrome P450. Corbin *et al.* [38] found that the derived coding region of human aromatase indicated without a doubt that this polypeptide belonged to a member of the cytochrome P450 super family of genes. The sequence contains regions of marked homology to those of other members, particularly an alleged membrane-spanning region, I-helix, Ozols and heme-binding regions. From these results they concluded that cytochrome $P450_{arom}$ belongs, like other steroidogenic P450 species, to a separate gene family within the super family. Corbin *et al.* [38] cloned human aromatase cDNA and expressed the enzyme in COS 1 monkey kidney tumor cells. The deduced amino acid sequence was similar in size to human placental aromatase and catalyzed the aromatization of androstenedione, testosterone and 16α-hydroxyandrostenedione. During the experiments, they were also able to inhibit the reaction with known aromatase inhibitors. The research group of Corbin *et al.* [38] concluded that the aromatization reaction appeared to be catalyzed by a single polypeptide encoded by the *CYP19* gene.

3.2 The CYP19 gene

The *CYP19* gene is located at chromosome 15q21.2 and consists of a 30 kilo bases (kb) coding region and a 93 kb regulatory region. The coding region of this gene, encoding human aromatase, spans nine exons commencing with exon II. Ten different tissue-specific promoters are found in the large regulatory region and are alternatively used in

different cell types. The common splice acceptor site seems to be the key mechanism behind the recruitment of such a large number of promoters. The activation of each promoter leads to splicing of an untranslated first exon onto this common junction directly upstream of the translation start site in the coding region [31,39,40].

Mahendroo *et al.* [40] and Simpson *et al.* [31] investigated the unusual model of alternative promoter usage in *CYP19*. Although different promoters of the CYP19 gene regulate the expression of aromatase in different tissues, an identical aromatase enzyme is expressed in all cells and tissue types [31,40]. The *CYP19* gene is very large and differs from other genes encoding cytochrome P450 enzymes in that many untranslated first exons are found in aromatase transcripts in a tissue-specific fashion. This phenomenon may be ascribed to tissue-specific promoters, which induce differential splicing. For example, the ovaries synthesize mostly estradiol, while the placenta produces estriol. Adipose tissue is responsible for synthesizing mainly estrone. The C_{19} steroid presented to the aromatase enzyme, in each tissue type, yields different tissue specific C_{18} steroids [31,40].

Aromatase expression in the granulosa cells of the ovary is controlled by the cAMP mediated gonadotropin, FSH. The proximal promoter, promoter II, that is situated directly upstream of exon II, is responsible for the expression of aromatase in the ovary [31,40]. In adipose tissue, adipocytes and adipose stromal cells express aromatase. Simpson *et al.* [31] found that aromatase expression and activity are much higher in the stromal cells that surround adipocytes. Transcripts from this tissue contain a distal, untranslated exon, I.4, located about 20 kb downstream from exon I.1, which means that it is 15 kb upstream from the start of translation. Mahendroo *et al.* [40] concluded from their results that a second transcript, with a different 5' terminus, is present in adipose tissue. These transcripts contain exon I.3 located 100 kb upstream of exon II. In contrast, placental transcripts contain an untranslated exon I.1 at their 5'–ends, located 40 kb upstream from the start of translation in exon II. A minor population of transcripts in the placenta contains exon I.2 in the 5'–untranslated region. As mentioned earlier, splicing of the untranslated exons occurs at a common 3'-splice junction upstream of the start of translation, to give rise to the identical mature transcripts [40]. This indicates that human

aromatase is encoded by a single-copy gene, regardless of the site of expression or the fact that different transcripts contain different 5'-termini (Fig. 3.2.1) [31,40].

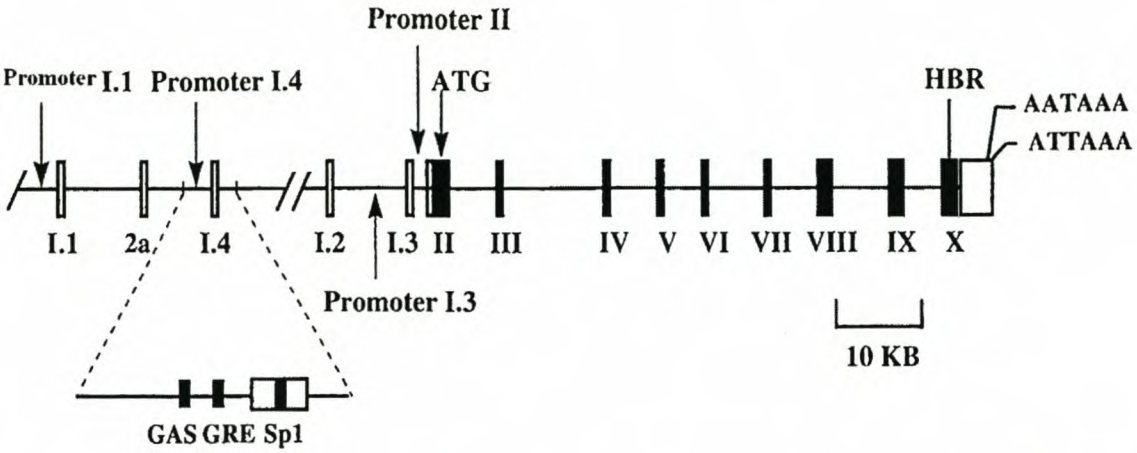


Fig. 3.2.1. The human *CYP19* gene. The septum in the open bar in exon II represents the common 3'-acceptor splice junction for the untranslated exons [31].

The physiological role of aromatase expression in adipose tissue still has not been fully elucidated. Some functions of aromatase activity in this tissue may include the maintenance of bone mineralization, thus preventing osteoporosis in both sexes, and the development of female reproductive organs. Inappropriate activation of abnormal promoters upregulates aromatase expression in estrogen dependent pathologic tissues such as breast cancer and endometriosis. Simpson *et al.* [31] used RT-PCR to examine local variations in aromatase expression in breast adipose tissue. They found that the highest expression of aromatase occurs in adipose tissue surrounding a tumor, as compared to expression distal to a tumor [31]. Mahendroo *et al.* [40] predicted that promoter I.4 is present only in breast tissue and that the other promoter found in adipose tissue, promoter I.3, is present in upper and lower body fat. In normal breast adipose tissue, aromatase expression is kept at low levels via promoter I.4, 73 kb upstream of the common coding region. In breast cancer, promoters II and I.3 activities are increased, and in addition the endothelial-type promoter I.7 is also upregulated [40]. From the results of Bulun *et al.* [39] it can be seen that, in estrogen-dependent malignancy breast cancer, four promoters are used for the expression of aromatase (II, I.3, I.7 and I.4) compared to

normal breast adipose tissue which makes almost exclusive use of only promoter I.4. The observations of Simpson *et al.* [31] and Bulun *et al.* [39] established that there is a definite form of communication between a breast tumor and the surrounding adipose cells in terms of the ability of the adipose cells to produce estrogens.

3.3 The mechanism of aromatase catalysis

As mentioned earlier, aromatase is a microsomal cytochrome P450-dependent enzyme that converts androgens to estrogens *via* three sequential oxidations and researchers have been able to isolate the 19-hydroxy and 19-oxo androgen intermediates formed in this reaction. The isolation of these intermediates suggested that the first two oxidation reactions occurred at the C_{19} carbon of the substrate. However, the mechanism of the C_{10}-C_{19} bond cleavage or the third oxidation has, to date, not been established. Several theories exist for the mechanism of the third oxidation. Korzekwa *et al.* [41], and others, have proposed two viable general mechanisms for the third oxidation step. The first proposal involves the addition of a peroxy intermediate to the aldehyde followed by homolytic or heterolytic bond cleavage. The second mechanism includes various intermediates involving the formation of a 1-radical intermediate. Korzekwa *et al.* [41] have used theoretical calculations to model reactions that involve the formation of 1-radical intermediates. These reactions also included the direct addition of the hydroxyl radical equivalent to the aldehyde group, followed by homolytic cleavage of the C_{10}-C_{19} carbon bond. When the hydroxyl radical was added to the aldehyde by direct addition, energy barriers of ~10kcal/mol were observed (Fig. 3.3.1). However, this hydroxy radical is extremely reactive and is therefore unlikely to serve as a good model for the active oxygenating species of a cytochrome P450-dependent enzyme. Very large energy barriers were found for the addition reaction when more stable radicals were used. This data suggested that the direct addition of the hydroxyl radical equivalent to the aldehyde is not a likely mechanism for carbon bond cleavage [41]. The ability of the functional group to stabilize the resultant radical correlates with the activation energies. The 2-ene-3-ol forms of the 1-radical appear to be favored over the keto forms. However, when there is competition with recombination to form the 1β-hydroxyandrogen, C_{10}-C_{19} cleavage from

the enol would be favored. The enol radical is much more stable than the ketone and this means a slower recombination rate. The A-ring of both the ketone and enol radical intermediates is bent below the plane of the steroid, while the estrogen products' A-ring is bent above the steroid plane. The geometries of the transition states for C_{10}-C_{19} cleavage are similar to the androgen substrates. This means that binding energy is likely to be lost during 1-radical formation and regained in the transition state of the cleavage reaction (Fig. 3.3.2) [41].

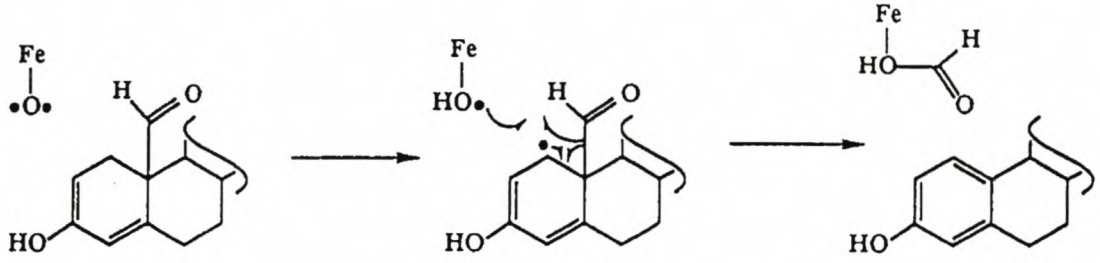


Fig. 3.3.1. 1-radical formation followed by direct addition of the hydroxyl radical equivalent to the aldehyde [41].

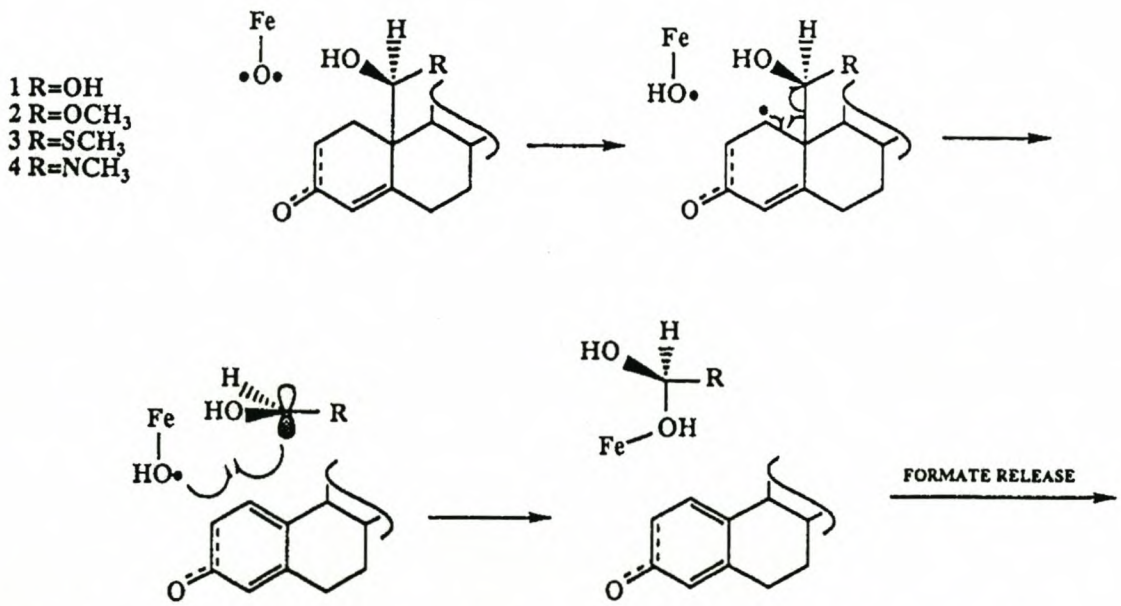


Fig. 3.3.2. Schematic representation of the C_1-radical formation followed by C_{10}-C_{19} bond cleavage [41].

Korzekwa *et al.* [41] did not model the alternative reaction of peroxy addition, however, other experimental evidence suggested that these peroxy intermediates are involved in cytochrome P450 mediated deformylation reactions. There are three general known mechanisms for the addition of a peroxy intermediate, one hemolytic and two heterolytic (Fig. 3.3.3). In the homolytic mechanism a 1β-hydrogen atom is transferred. In one heterolytic mechanism a proton is transferred while in the other a hydride is transferred [41]. These three mechanisms are concerted mechanisms with the disadvantage that *cis*-elimination of the 1β-hydrogen and 19-carbon must occur. However, most elimination reactions are transdiaxial [41]. These three transfer mechanisms involve the breaking and forming of three different bonds, which may lead to different stepwise mechanisms. Korzekwa *et al.* [41] considered the possibility of the generation of the diol in the active site in order for deformylation to occur. However, gem-diols have a low hydrophobicity and may therefore be poor substrates for cytochrome P450 implying that, if a diol is generated in the active site of the cytochrome P450 enzyme, the hydrogen abstraction/carbon-carbon cleavage reactions may take place as described earlier. From their results they concluded that the exact mechanism of the third hydroxylation step of aromatase was unclear [41]. No experimental evidence existed to indicate that the cytochrome P450 mediated deformylation reactions occur by hydrogen atom abstraction followed by carbon-carbon bond cleavage. The research group of Korzekwa *et al.* [41] suggested that more experimental data on α- and β-substituent effects would shed light on the true mechanism.

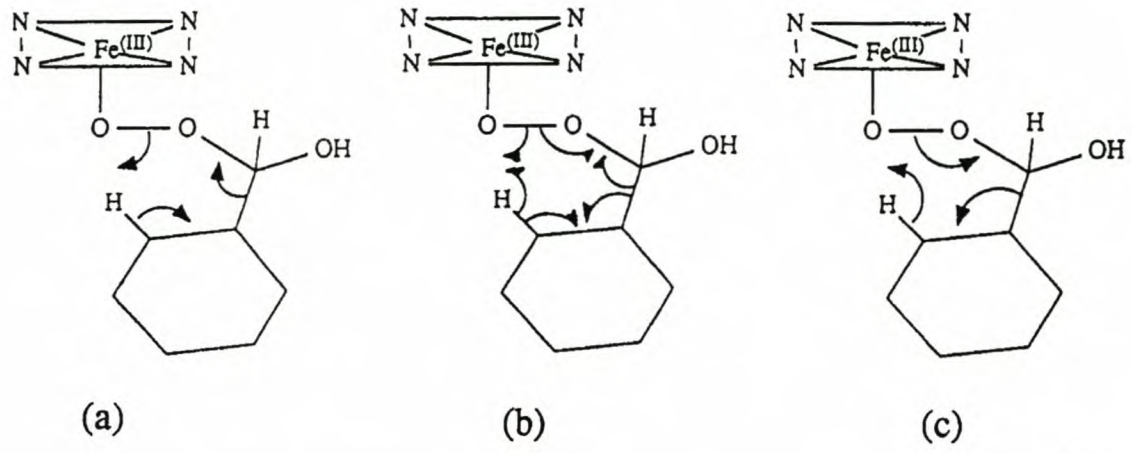


Fig. 3.3.3. Concerted mechanisms for the conversion of a peroxy addition intermediate to the deformylated product. A and C show the heterolytic mechanisms, where a proton and a hydride are transferred, respectively. B shows the homolytic mechanism where a 1β-hydrogen atom is transferred [41].

Akhtar *et al.* [42] were also studying the mechanistic properties of aromatase at the same time as Korzekwa *et al.* [41]. The conversion of androgens to estrogens is marked by replacing various methyl groups with hydrogen atoms. The removal of a methyl group is energetically unfavorable and thus unlikely. It is therefore more likely that the methyl group is oxidized to a carboxylic acid derivative where after it is removed as CO_2. Akhtar *et al.* [42] proposed that the carbon-carbon bond cleavage reaction is made possible by the involvement of the Fe^{III}-O-OH species, trapped by the electrophilic property of the carbonyl compound. This complex gives rise to a peroxide adduct producing an acyl-carbon cleavage by fragmentation (Fig. 3.3.4). Akhtar *et al.* [42] concluded from their results that aromatase shows catalytic pluralism, which is dependent on the electronic structure of the substrate. When a binary complex is formed from a substrate containing a C-H or C=C bond that is fittingly orientated, the hydroperoxide undergoes an oxygen-oxygen cleavage to yield the oxo-derivative. This derivative participates in further reactions. Alternatively, when the binary complex involves a carbonyl containing substrate, the Fe^{III}-O-OH species is intercepted by the electrophilic property of this group, providing the peroxide adduct that then fragments (3 in Fig. 3.3.5). According to Akhtar *et al.* [42], aromatase makes use of this acyl-carbon cleavage pathway. Aromatase must have evolved an active-site architecture that ensures a slow conversion of Fe^{III}-O-OH to

Fe^{IV}-O· for the bimolecular process, 1 – 3 in Fig. 3.3.5, to compete with it, permitting peroxide adduct formation [42]. Fig. 3.3.5 is a schematic representation of the proposal of Akhtar *et al.* [42] suggesting that the carbon-carbon bond cleavage reaction is made possible by the involvement of the Fe^{III}-O-OH species that is trapped by the electrophilic property of the carbonyl compound. Fig. 3.3.4 shows the acyl-carbon cleavage [42].

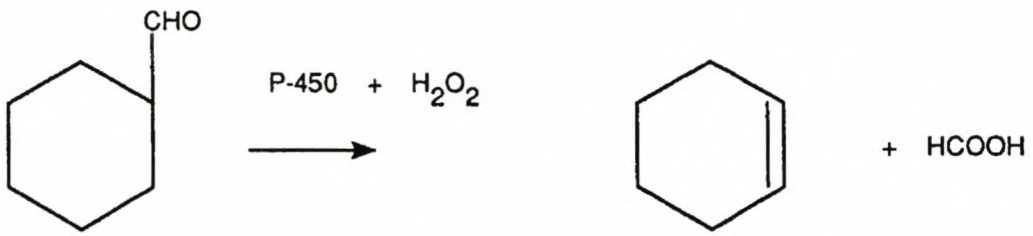


Fig. 3.3.4. Acyl-carbon bond cleavage mechanism [42].

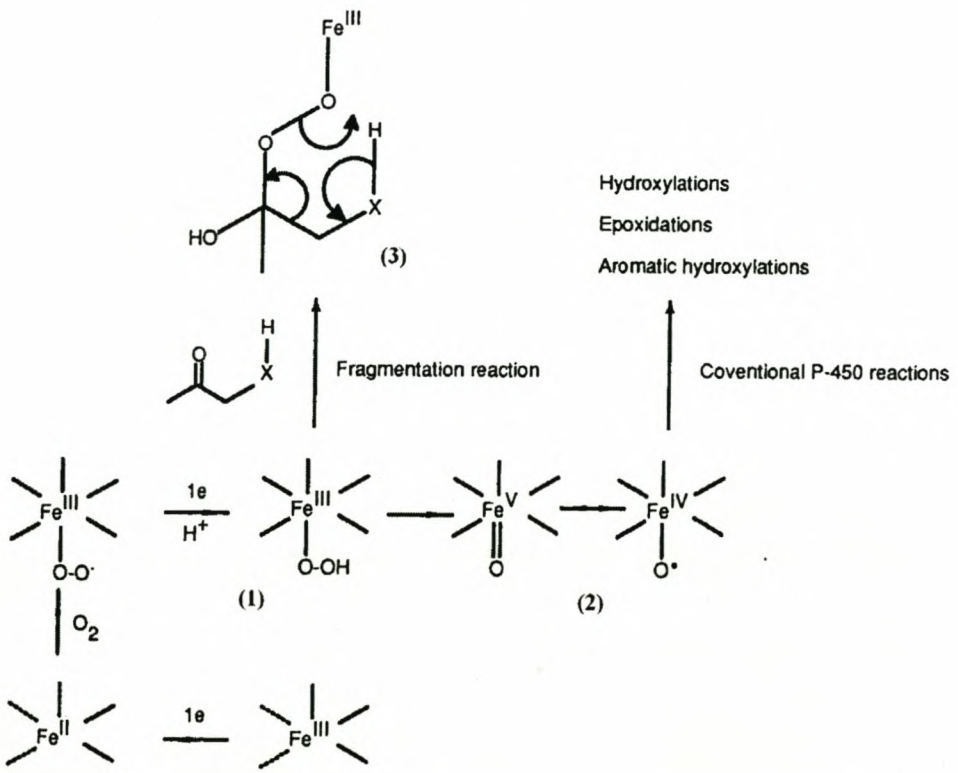


Fig. 3.3.5. Schematic representation of the carbon-carbon bond cleavage reaction, made possible by the involvement of the Fe^{III}-O-OH species that is trapped by the electrophilic property of the carbonyl compound [42].

The research group of Kao *et al.* [8] investigated the mechanism of aromatase using site-directed mutagenesis. As reported previously, the first two aromatase reactions are hydroxylations at position C19 of androgens. The oxygen activation of the third step leads to steroid ring A aromatization. The exact mechanism of the aromatization reaction remains to be confirmed. Kao *et al.* [8] proposed that a better understanding of the structure-function relationship of aromatase would provide the molecular basis for the reaction mechanism. The same laboratory also investigated the reaction mechanism by using five aromatase mutants to verify and refine their computer model. The prepared mutants were evaluated by inhibitory profile studies, enzyme kinetic analysis and reaction intermediate measurements. Glu302, Ser478 and His480 were indicated to potentially play an important role in catalysis and are likely to be closer to the active site than what was previously believed. Glu302 in the I-helix is considered an essential part of the active site of aromatase [8]. The results obtained by Kao *et al.* [8] suggested that the acidic group at this exact position is necessary for aromatase activity. A computer model showed that Glu302 is located near the C19 position of the substrate. As a result of this positioning, it was proposed that this residue is responsible for splitting the oxygen-oxygen bond which leads to the oxygen activation by forming an iron-oxo intermediate in the first hydroxylation step. Mutations of His480 to Lys led to higher activity than His480 to Gln, while having similar K_m values for androstenedione as the wild-type enzyme. This suggests that a positively charged amino acid is required at this position. However, these mutations result in lower estrone levels, which may mean that His480 plays an important part in aromatization and not so much in the first two hydroxylations. With His480 located close to C-3 and C-4 of the steroid, it may form a hydrogen bond with the C-3 keto group of the substrate and participate in the aromatization. His480 and Asp309 are two residues involved in the enolization at the 3-keto group of the 19-aldehyde androgen. The third step in the aromatase mechanism is suggested to be a peroxidative attack. Graham-Lorence and Peterson [43] and Kao *et al.* [8] suggested that the aromatization of the A ring follows the concerted mechanism as proposed by Coon *et al* [22].

For studies of the reaction where the 10β-methyl group of the androgen nucleus is removed by aromatase, Coon *et al.* [22] synthesized 3-oxodecalin-4-ene-10-

carboxaldehyde (ODEC) as an analog of the A and B rings of the 10β-formylandrogen intermediate. They found that this analog undergoes aromatization to yield 3-hydroxy-6,7,8,9-tetrahydronaphthalene (Fig. 3.3.6).

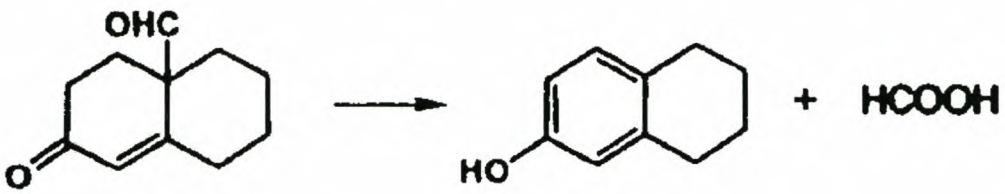


Fig. 3.3.6. Conversion of ODEC to hydroxytetrahydronaphthalene as catalyzed by P450 2B4 [22].

The conversion of this compound is similar to the steroid decarbonylation reaction in two ways. The deuterium in the formyl group of ODEC was preserved in the product, formic acid. The desaturation reaction was specific for the removal of the 1β-hydrogen atom. This shows that it is a stereospecific *cis* elimination of formate. Coon *et al.* [22] proposed the following concerted mechanism for the aldehyde deformylation (3rd step in aromatase mechanism) (Fig. 3.3.7) with 2-methylbutyraldehyde as a substrate. With NADPH present, an oxygen deprived cytochrome P450 heme iron-bound hydroperoxide reacts with the electrophilic carbonyl group of the 19-aldehyde forming an enzyme-bound peroxyhemiacetal-like intermediate. The double bond between C1 and C10 and formic acid would then be produced with rearrangement *via* a concerted mechanism, while alcohol, the alkane as well as formic acid can be produced by a stepwise mechanism with the help of a transient carbon radical [8,22].

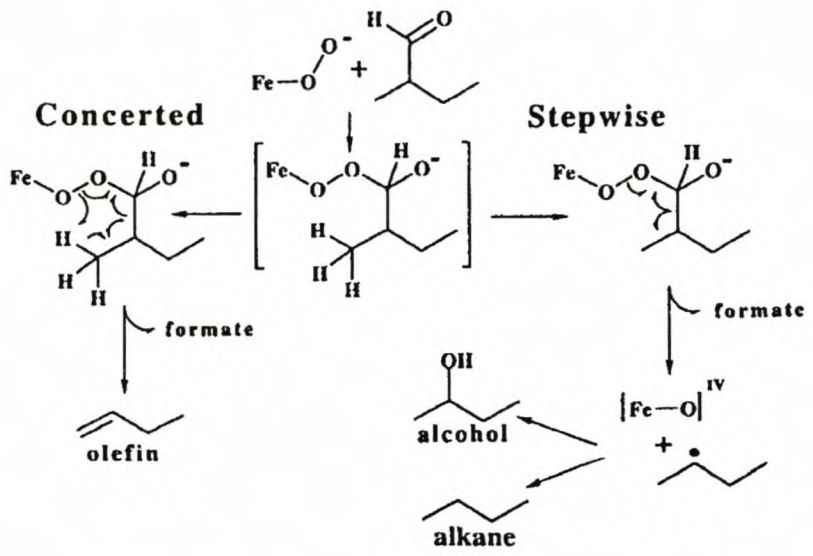


Fig. 3.3.7. Proposed concerted mechanism for the aldehyde deformylation as catalysed by aromatase, among other P450 enzymes. 2-methylbutyraldehyde was used as substrate [22].

The studies conducted by Kao *et al.* [8] and Coon *et al.* [22] have shed some light on the third aromatization step of aromatase. Computer-modeling, site-directed mutagenesis, inhibitor binding studies and intermediate profile analysis have played important roles in understanding the mechanism, however, more work must be done to completely unlock the mystery surrounding the third step of the aromatase reaction mechanism [8,22].

It is important to fully understand the catalytic mechanism of aromatase, since aromatase malfunction has been implicated in many diseases, especially breast cancer. If the mechanism is understood, the development of effective aromatase inhibitors will be facilitated.

3.4 Aromatase and breast cancer

Many women, especially in industrialized countries, suffer from breast cancer and other related cancers. With 1 million new cases of breast cancer diagnosed each year, it is an important risk factor that contributes to the morbidity and mortality of women around the world. Estrogens play an important physiological role in the human body. It is involved

in the development and maintenance of the female sexual organs, the reproductive cycle, reproduction, various neuroendocrine functions as well as the preservation of bone density. The normal mammary gland is directly affected by estrogen to promote growth and differentiation [44-46].

The release of pituitary prolactin is stimulated by estrogen. Prolactin acts on the mammary cell, supporting both normal mammary gland and tumor growth in the absence of ovaries and adrenals. Since estrogen cannot support mammary tumor growth in the absence of pituitary prolactin, some consider estrogen as probably being essential but not sufficient for the growth of certain tumors in breast tissue. Breast cancer is often hormone responsive, which means that the tumor contains estrogen receptors and requires estrogen for growth. In primary tumors, the values of estrogen receptors range from zero to 1000 fmol/mg of cytosol protein [48]. In 1974 an extensive study was performed to validate the suggestion made by Jensen and DeSombre [47] that the presence of estrogen receptors in malignant breast tissue might indicate that the tumor is hormone dependent. This observation may lead to the appropriate endocrine treatment with regression of the tumor. The results that McGuire *et al.* [48] obtained from their studies can be summarized as follows: Estrogen receptor values can be extremely useful in predicting results obtained with endocrine therapy. Patients, who had negative tumor estrogen receptor values, did not readily respond to endocrine therapy. A high percentage of patients with positive estrogen receptor values, responded favorably to endocrine treatment [48].

Estrogen has also been linked to the development of endometrial cancer. This disease usually occurs in women long after menopause is characterized by the elevated levels of estrogen production in nonendocrine tissues. A number of studies indicated that both estrogen and progesterone receptors were increased by estrogen administration in uterine tissues [49]. Since estrogen as well as progesterone levels fluctuate immensely during the menstrual cycle, progesterone must also be considered as a contributing factor to breast cancer. Huggins *et al.* [50-52] suggested that progesterone plays a role in stimulating tumor growth. The mechanism is, however unclear as it can either directly affect the tumor or modify the actions of other steroid hormones. It is possible that estrogen and progesterone exert feedback control on each other in the target tissue. Even after

considering progesterone, this steroid is still only a part of the complex hormonal control system that affects the growth and function of mammary cells. McGuire *et al.* [48] found that there are receptors present in MCF-7 cells for progestins, androgens, glucocorticoids and estrogens. Therefore, there is a complex interrelationship between various hormones that may or may not play a role in tumor growth; however, only estrogen, the product of aromatase, will be discussed further in detail.

3.4.1 Estrogens and cancer risk

All estrogens have three major distinguishable features namely an aromatic A ring, a phenolic hydroxyl group at the C-3 position and a methyl group at position C-13. Estradiol has a hydroxyl group at C-17, whereas estrone has a keto-group in the same position [11]. Fig. 3.4.1 is a schematic representation of the biosynthesis of estradiol from cholesterol with all the enzymes involved. Aromatase is responsible for the conversion of androstenedione to estrone as well as the production of estradiol from testosterone [12]. One third of all breast cancer patients have hormone-dependent breast cancer indicating that the tumor contains estrogen receptors and requires estrogen for further growth. Indeed, the case that one of the major known risk factors for breast cancer is associated with the extended exposure to elevated levels of estrogen [12,45,46,53-56]. Aromatase activity has been detected by many researchers in breast adipose tissue and breast cancer, implicating estrogens in the development of breast carcinomas [12,45,46,53-56]. Estrogens have also been implicated in other estrogen-dependent neoplasms such as endometriod, endometrial and surface epithelial-stromal ovarian carcinomas. The intratumoral aromatase possibly plays a role in converting any circulating androgens to estrogens, possibly with the aid of 17β-hydroxysteroid dehydrogenase (17βHSD) type 1 and estrogen sulfatase. This conversion may be an important mechanism of autocrine stimulation in these hormone-dependent tumors [12,45,46,53-56]. There are many other factors associated with the risk of cancer. Some of these risk factors include family history and genetic factors, mammographic density, endogenous and exogenous estrogens, bone density, age, diet and alcohol consumption [12,44,54,57].

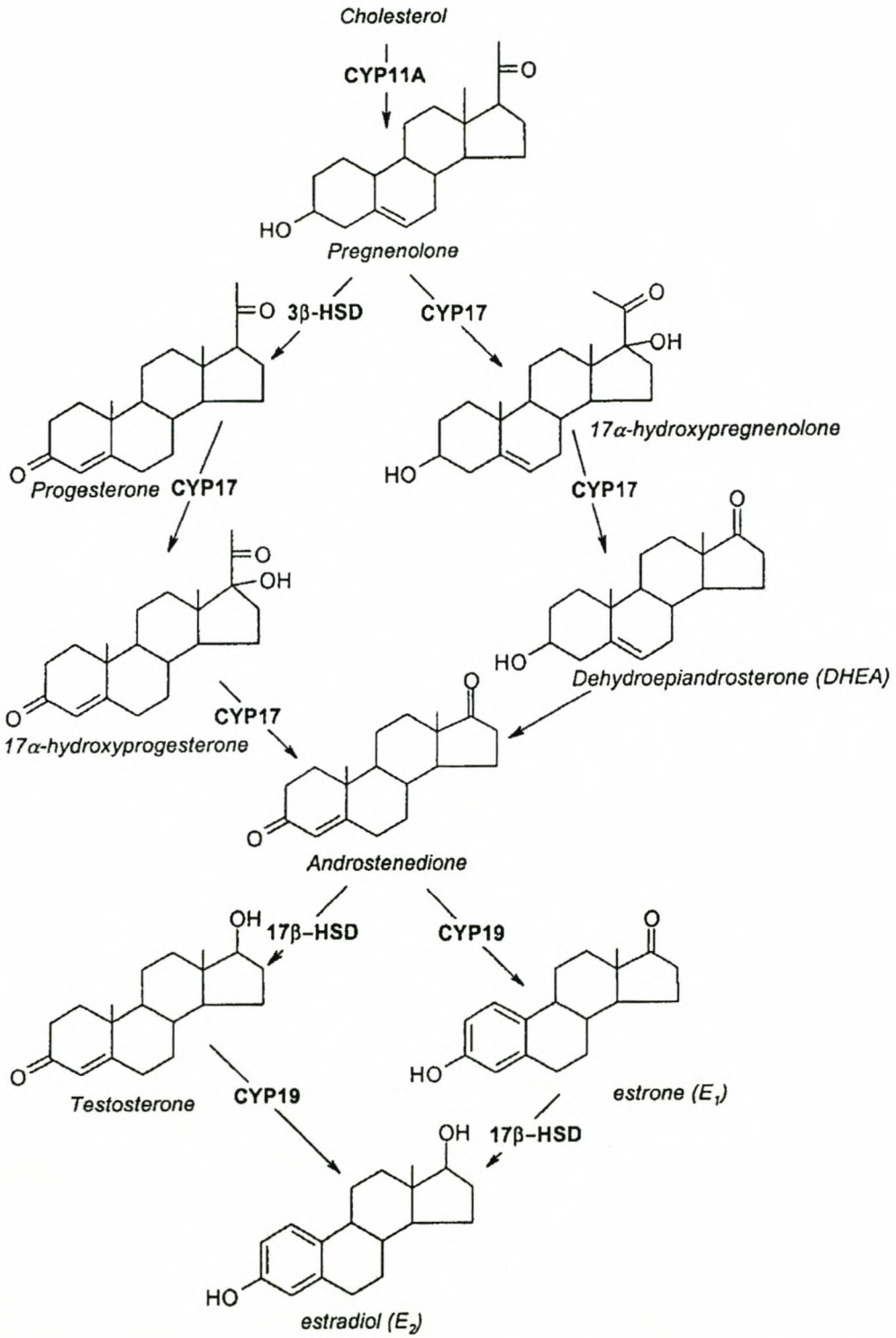


Fig. 3.4.1. Biosynthesis of estrogens from cholesterol. Androstenedione and testosterone are converted by aromatase to yield estrone and estradiol, respectively [12].

These risk factors are briefly listed below [12,44,54,57]:

Family history and genetic factors: BRCA1 was the first breast cancer susceptibility gene that was identified and has been linked to ovarian and breast cancer [12,44,54,57].

Mammographic density: A high percentage density in post-menopausal women put them at a four times greater risk of developing breast cancer than women with a lower percentage density [12,44,54,57].

Endogenous and exogenous estrogens: One of the first observations that suggested a hormonal role for estrogens in breast cancer, was bilateral oophorectomy. The earlier in life this procedure is carried out, the greater the risk reduction. It is also known that women with high bone density are at greatest risk of developing breast cancer. Since estrogens from any source is known to help retain bone mass, it is a possible to link bone density and breast cancer [12,44,54,57].

Age: Estrogen production increases as a function of aging and obesity. Another observation was that the younger the age at menarche and the older the age at menopause, the greater the risk of developing breast cancer. This observation suggests a direct relation between the period a woman is exposed to cycling ovarian hormones [12,44,54,57].

Diet: Some researchers have found a correlation between high fat intake and breast cancer risk, while others suggest diet only plays a role in children [12,44,54,57].

Alcohol consumption: Many groups have found a direct relation between an increased breast cancer risk and the consumption of more than three alcoholic beverages per day [12,44,54,57].

Although it is evident that there are many risk factors involved in breast cancer, however, estrogens seem to be a major complex contributor to the risk of developing breast, endometrial or ovarian cancer. Many research groups found a direct link between both the level and presence of aromatase activity and tumor cellularity [46,53,58]. These results suggest that cancer cells are the primary site of aromatase activity and that aromatase, and thus estrogens, contribute to the synthesis of a tumor, especially in post-menopausal women where ovaries are no longer the main source of estrogen production. In post-menopausal women, estrogens are produced in extra glandular tissues. These

estrogens may enter the bloodstream and subsequently on tumors *via* endocrine mechanisms [46,53,58]. Aromatase activity and expression, and hence the production of estrone is, however, the highest at the site of the malignant tumor or in areas proximal to the tumor. It has been found that aromatase activity is predominantly the highest in stromal cells. Tumors are made up of a large proportion of stromal cells that are close to the tumor epithelial cells. If the growth of hormonally responsive breast tumors is indeed stimulated by the *in situ* production of estrogen, paracrine interactions are required between the stromal and the epithelial compartments. Santner *et al.* [58] have shown that the stromal compartment contains most of the estrogen synthetic machinery, while the epithelial cells contain the estrogen receptors. Stromal aromatase may be the most important contributor to estrogen production in breast tumors, but the influence of other enzymatic sources, cannot be ruled out [45,46,55,58].

Estrone, formed by aromatase in peripheral tissues, is only weakly estrogenic and must be converted by 17βHSD to estradiol, the most potent estrogen, to exert its effects. 17βHSD has been detected in breast tumors together with estrone sulfotransferase, which converts estrone to estrone sulfate [54,55,58]. Brueggemeier *et al.* [45] found a linear correlation between the expression of aromatase and cyclooxygenases (*COX-1* and *COX-2*) in breast cancer specimens. In breast cancer, both these enzymes are regulated by complex paracrine interactions, resulting in significant consequences on the pathogenesis of breast cancer [45,54,55,58].

Miller [46] found that estrogen receptors also play an important role in breast tumors. However, estrogen biosynthesis can be detected in both estrogen receptor-positive and negative cancers, but is more likely to be of significance in estrogen receptor-positive tumors. Miller [46] postulated two possible explanations why increased levels of aromatase are found in the adipose tissue of breast cancer patients. Firstly, it is possible that the high activity of aromatase leads to elevated levels of estrogen, which promotes cancerous growth at that specific site. The second reason might be that the malignant tumors are secreting factors into the local environment that can either be responsible for the induction or the stimulation of aromatase activity. Therefore, it is likely that the cancerous tumor might induce the activity of aromatase in adjacent breast adipose tissue

where higher levels of estrogen receptor-α is also detected. Estrogen receptor-α is thought to mediate cellular proliferation.

Esteban *et al.* [53] have found it difficult to relate the functional significance to locally produced estrogens in the absence of estrogen-receptors, since no correlation was seen between intratumoral estrogen production, aromatase activity, as well as estrogen and progesterone receptors. It is possible that estrogen binds to different receptors than those detected by their experimental methods [44,46,53]. Another receptor, estrogen receptor-β, has also been identified in various estrogen target tissues as well as normal and malignant breast tissue. However, this receptor has different binding affinities and tissue distribution compared to estrogen receptor-α. Estrogen receptor-α levels increase and decrease with the menstrual cycle, while the levels of estrogen receptor-β remain constant, and are high from birth to adulthood. Maintaining a constant level may regulate the proliferation of cells negatively, which in turn may have a protective role in normal breast tissue. Estrogen is responsible for down-regulating estrogen receptor-α levels and some breast cancers might be a result of the inability of estrogen to fulfill this regulatory role. During breast as well as ovarian carcinogenesis, estrogen receptor-α is over expressed at much higher levels than estrogen receptor-β, which seems to be down regulated [12,44,56,59,60]. Maggiolini *et al.* [56] suggested that the ratio of estrogen receptor-α/β, together with determination and detection of intratumoral aromatase, may be a solution to monitoring the potential responsiveness of hormone-dependent cancers.

The question whether or not only estrogens will initiate breast cancer remains controversial. Proliferation of normal and malignant breast cells take place when these cells are exposed to estrogens. In the case of the malignant cells, the proliferation of the cells explains why estrogens increase breast cancer risk. A high rate of cellular proliferation in normal cells may lead to the accumulation of DNA adducts and ultimately mutations, since the cells have less time to repair DNA damage. Hilakivi-Clarke *et al.* [44] thus proposed that estrogens may initiate breast cancer only once the DNA repair mechanisms are damaged. Mitrunen *et al.* [12] reported that rapid cell proliferation lead to a greater chance of genetic error. Only once mutations have been introduced into the cell, can estrogens enhance the replication of the cells carrying these

mutations. Estrogens induce free-radical-mediated DNA damage, some of these damages include single-strand DNA nicks, an increase in 8-hydroxyguanine formation, estrogen-DNA adducts, lipid peroxidation and protein oxidation. Thus, many researchers suggest that the DNA repair enzymes may influence the risk of cancer [12,44].

Other factors may also influence the occurrence of breast cancer. Maggiolini *et al.* [56] identified several transcriptional enhancers secreted from breast carcinomas, which control aromatase expression in the malignant tissue. They also found that the overexpression of aromatase is associated with different coding start sites or alternative splicing events in exon I. Maggiolini *et al.* [56] concluded that aromatase inhibitors could prove to be an adjuvant therapeutic strategy for the treatment of hormone dependent breast tumors, especially in post-menopausal women.

3.4.2 Gene expression, promoter usage and polymorphisms in cancer

As mentioned before, Bulun *et al.* [39] reported that, in estrogen-dependent malignancy breast cancer, four promoters are used for the expression of aromatase (II, I.3, I.7 and I.4). In normal breast adipose tissue promoter I.4 is used almost exclusively. Brueggemeier *et al.* [45] also observed that, when aromatase expression levels are elevated in breast cancer tissues, a switch occurs in the major promoter region utilized with promoter II, specific for the ovary, testis and prostate, being used predominantly. When Utsumi *et al.* [61] investigated the relationship between aromatase mRNA levels and the alternative use of multiple exons 1 in human breast tissue, they reported that when this switch of promoter I.4 to I.3 occurs, it leads to increased levels of aromatase mRNA in some patients. The mRNA that is transcribed from promoter I.3 in breast cancer exhibits a higher incidence of involved nodes than those transcribed from promoter I.4 [61]. The regulation of estrogen biosynthesis is normally controlled by glucocorticoids and cytokines. However, when a promoter switch is observed, the control changes to the cAMP-mediated pathway. Intracellular cAMP levels are increased by the prostaglandin PGE_2 and leads to stimulation of estrogen biosynthesis. Increased *COX-1* and *COX-2* gene expression results in enhanced COX enzyme levels. The elevated enzyme levels lead to an increased production of prostaglandins, ultimately stimulating aromatase expression and activity [45].

There is paracrine interaction between stromal adipose cells and carcinoma cells and this interaction might cause a change in the aromatase mRNA levels in breast cancer. Inductive or suppressive factors, which are possibly secreted by carcinoma or nonmalignant cells, may account for the difference of aromatase mRNA expression levels. These factors can regulate the expression levels through interaction with the promoter region of the *CYP19* gene. Studies by Utsumi *et al.* [61] could not clarify whether the increased levels of aromatase mRNA observed in cancerous tissue are a consequence or cause of carcinogenesis. During carcinogenesis, the surrounding breast stromal cells are under severe physiological conditions which may cause the promoter switch of the human CYP19 gene and hence the abnormal expression thereof [61].

Haiman *et al.* [62] examined the relationship between breast cancer risk and genetic polymorphisms in the *CYP19* gene. Previous research showed that there is a greater frequency of tetranucleotide $(TTTA)_n$ repeat alleles in intron 4 among women affected with cancer. No association between C to T single nucleotide polymorphism (SNP) in exon 10 of CYP19 and breast cancer risk was, however, observed. Haiman *et al.* [62] were also unable to prove that the association between breast cancer risk and rare tetranucleotide repeat alleles was accounted for by this SNP in exon 10. Unlike Haiman *et al.* [62], Berstein *et al.* [63] reported that there was a significant association between the level of aromatase mRNA and the presence of this SNP in exon 10 in breast tumors. They focused on the initiation and promotion of endometrial cancer and the influence of estrogen on these processes [63]. In women with endometrial cancer, the A2/A2 CYP17 genotype is under-represented while CYP19 genotypes with the longest A6 and A7 alleles are over-represented [63]. These results indicated that higher aromatase activity is detected in females with the highest risk of endometrial cancer. Women at high risk are usually carriers of the A1/A1 and A1/A2 CYP17 genotypes and A6/A6 or A6/A7 CYP19 genotypes. Normal human endometrium tissue does not express aromatase and it is only detected in endometrium cancer tissue [63]. The results obtained by Bernstein *et al.* [63] suggested that a 'high risk' CYP19 or CYP17 genotype transforms into a 'high activity' phenotype of the tumor. This increases local estrogen production resulting in further tumor growth. In depth analysis of intratumoral aromatase in tumors is therefore

important to gain a better understanding of development and biological behavior of tumors and the clinical management of patients [53,55,56].

3.5 Summary

The *CYP19* gene is located at chromosome 15q21.2 and consists of a 30 kb coding region and a 93 kb regulatory region. The product of this gene is known as the enzyme aromatase. This enzyme is essential for the biosynthesis of estrogens from androgens and it is unique in that it catalyses three sequential hydroxylations to produce an aromatic C18 estrogen. The first two reactions are hydroxylations that take place on position C19 of androgens that serve as substrates for aromatase. The oxygen activation in the third step leads to aromatization of the steroid A-ring. All three these reactions are carried out at the same active site of the single aromatase enzyme complex. There are ten different tissue-specific promoters in the large regulatory region of the *CYP19* gene and they are alternatively used in different cell types.

Aromatase activity has been detected by many researchers in breast cancer, implicating that estrogens play a role in the development of breast carcinomas. Estrogens play an important physiological role in the human body. All estrogens have three major distinguishable features namely an aromatic A ring, a phenolic hydroxyl group at the C-3 position and a methyl group at position C-13. Estradiol has a hydroxyl group at C-17, whereas estrone has a keto-group at the same position.

In estrogen-dependent malignancy breast cancer, four promoters are used for the expression of aromatase (II, I.3, I.7 and I.4). In normal breast adipose tissue almost only promoter I.4 is used. Many researchers have reported that when a switch of promoter I.4 to I.3 occurs, it leads to increased levels of aromatase mRNA levels in some patients.

While stromal aromatase may be the most important contributor to estrogen production in breast tumors, the influence that other enzymatic sources might have, has also been investigated. Many research groups suggest that DNA repair enzymes play a major role in the risk of cancer. When DNA repair enzymes are not functioning as well as they should, mutations can easily be overseen during cell proliferation.

Human cytochromes P450 only occur in low concentrations in tissues, making them extremely difficult to study. However, when these proteins are heterologously expressed, it becomes easier to determine the enzyme activities in a foreign environment, e.g. yeast, as well as determining the structure/function relationships associated with cytochromes P450 by site-directed mutagenesis. Expression of human cytochromes P450 in heterologous expression systems could also aid in the evaluation of xenobiotics' effect on these enzymes and provide sufficient pure cytochrome P450 proteins for antibody production, enabling the localization of aromatase in breast tissue, for example.

Human aromatase has been successfully expressed in COS 1 monkey kidney tumor cells [38], in mammalian cells such as breast cancer cells (MCF-7) [64], in insect cells using a baculovirus mediated system [9,65,66], in *E. coli* [10,67] and the protogynous wrasse, *Halichoeres tenuispinis* [68]. In this study, we have successfully expressed the human aromatase enzyme in the yeast *P. pastoris*. Much research still needs to be done on the levels, mechanism of action and activity of aromatase in breast cancer. An economic and effective method for the production of high yields of aromatase will therefore be essential for further immunological and enzymological studies on this extremely important and fascinating protein.

CHAPTER FOUR

The expression of human *CYP19* in *P. pastoris*

4.1 Introduction

The mRNA encoding human aromatase has been localized in peripheral tissues in previous studies [55,70]. Aromatase protein levels in these tissues have to date, not been determined and there is still much controversy over the exact localization of this protein in breast tissue (stroma vs. epithelia) [69,70]. Breast, endometrium and ovarian cancers contribute significantly to female mortality rates worldwide [11,39,40,45-48,53,58] and aromatase activity, catalysing the production of estrogens from androgens, has been proved to be linked to the above-mentioned cancers [11,39,40,45-48,53,58]. In hormone-dependent cancers, a continuous supply of estrogen is necessary for the initiation and promotion of the tumor. The human *CYP19* gene is tissue-specific and under the control of alternative promoters by the means of alternative splicing mechanisms [11,39,40,45-48,53,58].

The aim of this study was to express the human *CYP19* gene in *P. pastoris* at high yields to raise an antibody against for immunohistochemical studies that could ultimately lead to the determination and localization of aromatase in normal and cancerous breast tissue *via* enzyme linked immunosorbent assay (ELISA) and immunocytochemistry.

P. pastoris is a eukaryotic methylotrophic yeast, capable of metabolizing methanol as its sole energy and carbon source. This expression system provides all the advantages of higher eukaryotic expression, such as protein processing and folding as well as posttranslational modification. The oxidation of methanol to formaldehyde is catalysed by the enzyme alcohol oxidase (Aox1p), utilizing molecular oxygen. This reaction generates hydrogen peroxide that is very toxic to the cell. To avoid cell death, methanol metabolism takes place in the peroxisomes [72-75]. The pPIC3.5K vector is a plasmid designed to identify multiple integrations of a gene in the *P. pastoris* genome *in vivo*. This 9004 bp plasmid provides intracellular expression of the gene of interest. It has five

unique restriction sites in the multiple cloning site and offers *HIS4* selection in *P. pastoris* [74]. Fig. 4.1.1 is a schematic representation of the pPIC3.5K vector.

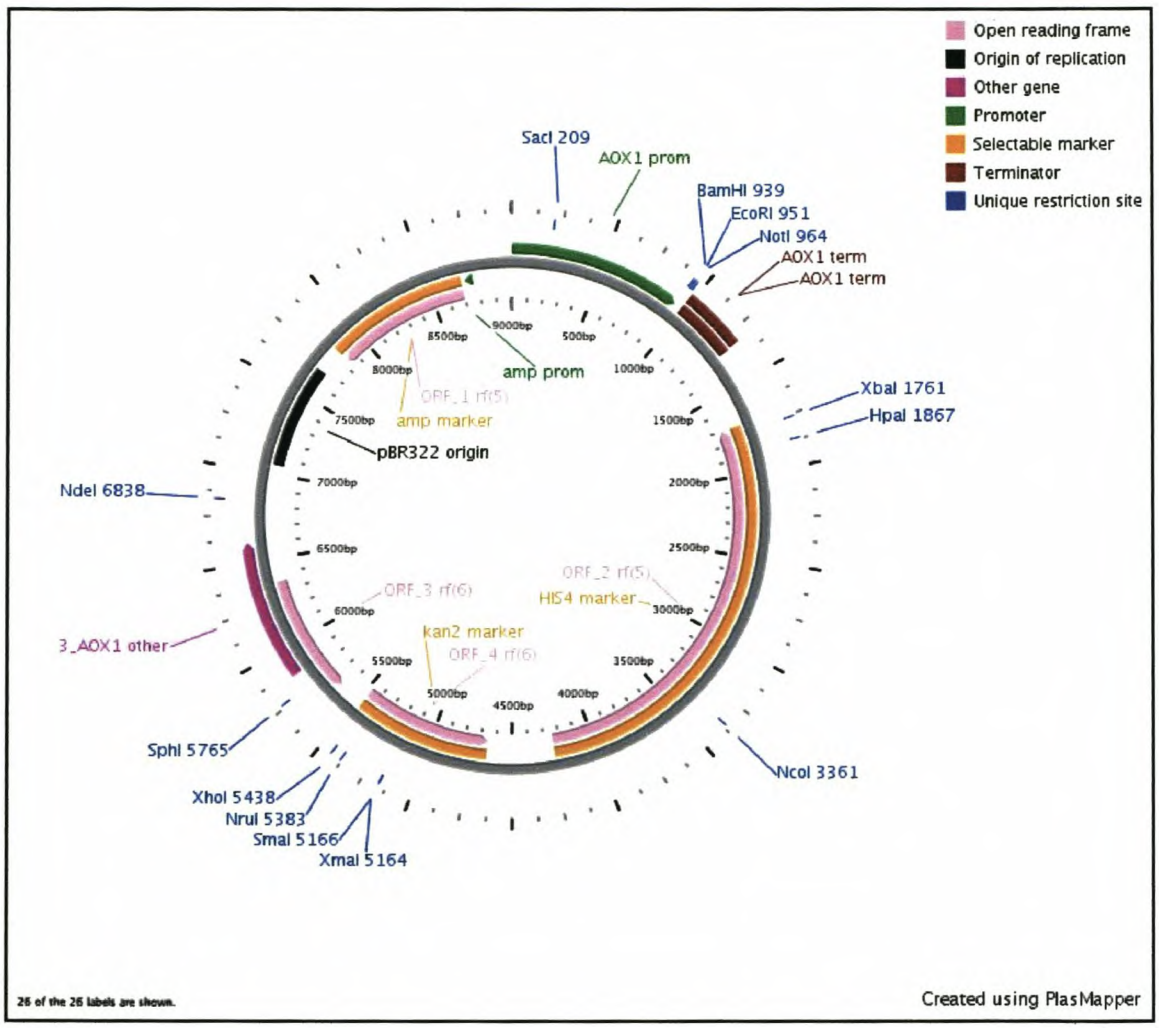


Fig. 4.1.1. Map of expression plasmid pPIC3.5K[1]. *5'AOX1*, ***P. pastoris AOX1* gene promoter**; *3'AOX1(TT)*, ***AOX1* transcription terminator**; *HIS4 ORF*, ***P. pastoris* histidinol dehydrogenase gene**; *3'AOX1*, ***3'AOX1* downstream sequence**; ColE1, **origin of replication**; *Ampicillin*, **ampicillin resistance gene**; Kanamycin, **geneticin resistance gene**.

[1] **Xiaoli Dong, Paul Stothard, Ian J. Forsythe, and David S. Wishart "PlasMapper: a web server for drawing and auto-annotating plasmid maps" Nucleic Acids Res. 2004 Jul 1;32(Web Server issue):W660-4.**

4.2 Cloning of CYP19 into the pPIC3.5K expression vector

4.2.1 Materials and methods

Unless otherwise specified, all reagents were purchased from Sigma and Merck. The pGEM-T vector, containing the human *CYP19* gene and the pPIC3.5K vector, was located in *E. coli* strain JM109 (Promega) cells. The *CYP19* gene, coding for six N-terminal histidine residues, was previously cloned into the pGEM-T vector in our laboratory to yield pGEM-T-CYP19. Sterile Luria-Bertani (LB) medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl, pH 7.0 adjusted with NaOH) was used for the routine growth in *E. coli*. Selection of bacteria transformants was carried out on LB-agar (15 g/L), containing ampicillin (100 µg/ml).

4.2.2 Isolation of pGEM-T-CYP19 and pPIC3.5K

Cultures of *E. coli* (100 ml), containing pGEM-T-CYP19 and pPIC3.5K, were grown overnight in LB medium at 37°C while shaking at 175 rpm (Innova shaking incubator, New Brunswick). The plasmids were isolated with the Nucleobond® PC100 plasmid preparation (Macherey-Nagel) kit, according to manufacturer's specifications.

4.2.3 Purification of pPIC3.5K and CYP19 cDNA

4.2.3.1 Restriction digestion

pGEM-T-CYP19 (1 µg x 12) and pPIC3.5K (1 µg x 12) were restriction digested, for 120 min at 37°C, each in a final volume of 25 µl containing 2.5 µl SuRE/Cut Buffer H (supplied) and NotI (1 U), (Roche). DNA loading buffer (0.1% Orange G (w/v), 20% Ficoll (w/v), 10mM EDTA, pH 7) was added to the restriction digested pGEM-T-CYP19 vector. The DNA fragments were separated by electrophoresis (55 V) on a low-melting agarose gel (1%), in TAE buffer (40 mM Tris-acetate, 2 mM $Na_2EDTA.2H_2O$ and 20 mM acetic acid), for 3 h at 4°C. Gels were stained with ethidium bromide (0.5 µg/ml) for 20 min. The stained DNA fragments were visualized on a UV transilluminator. The CYP19 DNA band was excised and purified with the gel extraction kit (Qiagen Inc.), according to manufacturer's specifications. The digested pPIC3.5K vector was purified

with the Wizard™ DNA clean-up kit (Promega), according to the manufacturer's specifications.

4.2.3.2 Dephosphorylation

The linearised pPIC3.5K vector was dephosphorylated prior to ligation to prevent religation of the vector in downstream reactions. The dephosphorylation mixture (30 µl) containing DNA (6 µg), 3 µl reaction buffer (50 mM Tris-HCl, pH 9.3, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine) and calf intestinal alkaline phosphatase (CIAP) (2.03 U) (Promega) was incubated at 37°C for 30 min. An additional 2.03 U CIAP was added and the reaction mixture incubated for 30 min. EDTA (2 µl, 0.5 M) was added and the resultant reaction mixture incubated at 65°C for 20 min to terminate the dephosphorylation reaction. The DNA was purified with the Magic DNA Clean-Up® kit (Promega), according to the manufacturer's specifications.

4.2.3.3 Ligation

The purified CYP19 cDNA was subsequently ligated using 1:1, 1:3 and 1:5 molar ratios of vector: insert DNA. DNA insert (200 ng) and vector (200 ng) was used for the 1:1 ratio in a 10 µl ligation reaction containing T4 DNA ligase (1 U) (Roche), 3 µl ligation buffer (supplied) and ATP (20 nM). The reaction mixture was incubated at 4°C, overnight.

4.2.4 Transformation of *E.coli* strain JM109 cells with pPIC3.5K-CYP19

E. coli strain JM109 (200 ml) was grown overnight in LB media, shaking at 175 rpm, at 37°C. Competent cells were prepared as described in the calcium chloride procedure by Sambrook *et al.* [76]. The competent cells were transformed with the construct and grown on LB-plates supplemented with ampicillin (100 µg/ml). Positive transformants were selected and grown overnight in LB media (5 ml) supplemented with ampicillin, and the plasmids were purified with the Wizard™ Plus SV mini preparation kit (Promega), according to manufacturer's instructions. A pPIC3.5K-CYP19 positive clone was inoculated in LB media (100 ml), as described previously. The pPIC3.5K-CYP19

construct was isolated using the Rapid midi preparation kit (Life Technologies), according to manufacturer's specifications. The nucleotide sequence of CYP19 gene fragment in pPIC3.5K-CYP19 was verified (University of Stellenbosch DNA sequencing facility) using specific primers, two in the forward direction and three in the reverse direction. The upstream primers were 5'AOX1P and AromB1 and the downstream primers were 3'AOX1, AromA2 and AromB2. Table 4.1 represents the sequences of the primers used for the DNA sequencing of the CYP19 fragment.

Table 4.1. Table showing the sense and antisense primers' sequences used.

Primer	Sequence
5'AOX1P	5'-GACTGGTTCCAATTGACAAGC-3'
AromB1	5'-GTCACAGTCTGTGCTGAATC-3'
3'AOX1	5'-GCAAATGGCATTCTGACATCC-3'
AromA2	5'-CTCCAACCTGTCCAGATGTG-3'
AromB2	5'-GCACTGGTTCACATTCTCTC-3'

The plasmid was also subjected to polymerase chain reaction (PCR) to amplify the gene using the 5'AOX primer and the 3'AOX primer. Each PCR amplification mixture (50 µl) contained pPIC3.5K-CYP19 DNA (400 ng), 200 µM each of dATP, dTTP, dCTP and dGTP, 2.5 µM each of the 5'AOX and 3'AOX primers and Super-therm DNA Polymerase (2.5 U, Abgene). The following procedure was carried out in a PCR Sprint (Hybaid): (1) denaturation at 94°C for 2 min; (2) 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min; and (3) a final extension at 72°C for 7 min. DNA loading buffer (5µl), was added to 10µl of each PCR product mixture prior to electrophoresis. The PCR product was analyzed by agarose gel electrophoresis as described in section 4.2.3.1.

4.3 Transformation and homologous recombination in GS115 strains of P. pastoris

4.3.1 Introduction

Uptake of DNA is hindered by the yeast cell wall. It is therefore necessary to either partially remove the cell wall, or to create pores to facilitate DNA uptake during transformation. The transformation of *P. pastoris* cells with foreign DNA is less efficient than that of *Saccharomyces*. Nevertheless, electroporation and spheroplasting provide the highest efficiency of transformation (up to 10^3 to 10^4 transformants per μg DNA). For this reason, both techniques were used in this study to determine which method provided the highest copy number of the *CYP19* gene integrated into the *P. pastoris* genome.

4.3.2 Materials and methods

P. pastoris strain GS115 (*his*4) (Invitrogen), used for the expression of human cytochrome aromatase, were grown, transformed and analysed according to the manufacturer's instructions. Yeast extract peptone dextrose (YPD) media (1 % yeast extract, 2 % peptone, 2 % glucose) was used in the routine growth of yeast. Selection of yeast transformants was carried out on minimal dextrose (MD) plates and contained: 13.4 g yeast nitrogen base without amino acids, 400 μg biotin, 20 g dextrose and 15 g agar per liter. Minimal methanol (MM) plates contained 13.4 g yeast nitrogen base without amino acids, 400 μg biotin, 5 ml methanol and 15 g agar per liter. Regeneration dextrose base (RDB) plates contained 1 M sorbitol, 2 % dextrose, 1.34 % yeast nitrogen base without amino acids, 4 x 10^{-5} % biotin, L-glutamic acid, L-methionine, L-lysine, L-leucine, L-isoleucine each at 0.005 % and 2 % agar. For heterologous expression *P. pastoris* was grown in buffered minimal glycerol (BMGY) medium (0.1 M potassium phosphate pH 6.0, 1.34 % w/v yeast nitrogen base without amino acids, 4 x 10^{-5} % biotin, 1 % v/v glycerol) or in buffered minimal methanol medium without glycerol, containing 0.5 % v/v methanol (BMMY).

4.3.2.1 Spheroplasting

P. pastoris GS115 cells were grown and prepared for spheroplasting as described by the manufacturer [74]. Plasmid DNA (20 µg) was linearized with SacI (Promega). *P. pastoris* GS115 (*his*4) was transformed by the spheroplasting method as previously described [74]. The integration of the *CYP19* gene into the *P. pastoris* genome was monitored as described in section 4.3.2.3.

4.3.2.2 Electroporation

Plasmid DNA (15µg) was linearized with SacI. *P. pastoris* GS115 (*his*4) was transformed by electroporation (Bio-Rad GenePulser) as previously described [74]. After transformation the integrity of yeast cells was monitored as described in section 4.3.2.3 below.

4.3.2.3 Verification of integrity of yeast cells after transformation

Transformed yeast cells were incubated on minimal dextrose RDB-agar plates (histidine deficient plates). The plates were incubated for 4 days at 30°C to select for His^+ recombinants. Yeast colonies were transferred to YPD-agar (histidine deficient) plates containing G418 (Sigma) added at 0.25 and 0.50 mg/ml. The level of G418 resistance correlates with the number of kanamycin resitance genes integrated into the *P. pastoris* genome. A single copy of pPIC3.5K plasmid integrated imparts resistance to G418 at 0.25 mg/ml. Colonies were selected and transferred to either dextrose (MD) or methanol (MM) minimal medium as a carbon source to determine the methanol utilization phenotype of the cells. Transformants that displayed the ability to grow on both carbon sources were selected for further evaluation and small-scale expression studies. The presence of cDNA encoding aromatase integrated into the yeast genome was confirmed by direct PCR analysis (PCR Sprint, Hybaid) applying sense and antisense oligonucleotides to amplify the *CYP19* gene. This protocol was adapted from a method as described by Linder *et al.* [77]. Colonies were selected from MD plates, suspended in sterile water (30 µl) and lyticase (100 U) (crude, from *Artrobacter luteus*; Sigma) was added and incubated for 15 min at 30°C. The digested cells were frozen in liquid nitrogen (1 min) and the lysate was used for PCR amplification. Each PCR amplification mixture

(50 µl) contained DNA/cell lysate (5 µl), 200 µM each of dATP, dGTP, dTTP and dCTP, 5'AOX1 and 3'AOX1 primers, each at 2.5 µM and Super-therm DNA Polymerase (2.5 U) (Abgene) in reaction buffer containing $MgCl_2$ (25mM). The amplified DNA fragment was analysed by agarose gel electrophoresis as described in section 4.2.3.1.

Single colonies containing the verified recombinants in *P. pastoris* GS115 were grown in MD (10 ml) overnight at 30°C, shaking at 275 rpm. Cells were isolated by centrifugation at 3000 x g for 5 min at room temperature and resuspended in fresh, sterile MD containing 15 % glycerol. Cells were frozen in liquid nitrogen and stored at -80°C.

4.3.2.4 Expression of aromatase in *P. pastoris*

Several clones of the recombinant strains of *P. pastoris* were screened for adequate expression levels of the CYP19 gene. *P. pastoris* strains GS115/pPIC3.5K-CYP19 and GS115/pPIC3.5K were and incubated on YPD medium at 30°C until single colonies appeared. Single colonies were collected from the different strains and grown for 18 h in BMGY (25 ml), shaking at 275 rpm at 30°C, until the cultures reached an optical density between 2 and 6 at 600 nm (OD_{600}). The cells were harvested by centrifugation at 3500 x g for 5 min at room temperature. The cell pellet of each clone was resuspended to an approximate OD_{600} of 1 in BMMY (200 ml), covered with sterile gauze and incubation was continued at 30°C with shaking at 275 rpm. Methanol was added after every 24 h to a final concentration of 0.5% (v/v). Aliquots (1 ml) of the culture media were removed at selected time intervals i.e. 0, 24, 48, and 60 h to determine the optimal time for expression. Cells were harvested by centrifugation at 3500 x g and the resultant pellets were stored at -80°C. The fractions were analysed for the presence of intracellular heterologous aromatase proteins by SDS-PAGE (Coomassie staining) and immunoblot analysis.

4.3.2.5 Screening for intracellular expression of aromatase

The cell pellets were thawed on ice and resuspended in 100 µl Breaking Buffer (BB, 50 mM NaH_2PO_4, pH 7.4, 1 mM PMSF, 1mM EDTA and 5 % glycerol). All subsequent steps were carried out at 4°C. The suspension was transferred to a 1.5 ml microcentrifuge

tube and centrifuged at 3000 x g at 4°C for 10 min. The washed cells were resuspended in BB (50 µl) and an equal volume (cells and buffer) of acid-washed glass beads (0.5 mm) was added to each sample. The samples were vortexed for 30 s and placed on ice for 30 s for 8 cycles. After completion of the cycles, the samples were subsequently centrifuged at maximum speed in a bench top microcentrifuge for 10 min at 4°C. The clear lysate was collected and stored at -20°C.

4.3.2.6 Analysis by SDS-PAGE and Western blot verification

SDS-PAGE was carried out according to the method of Laemmli [78] using an acrylamide concentration of 10 % (v/v). Rainbow colored protein molecular weight markers (Amersham Pharmacia) consisting of myosin (220 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21 kDa) and lysozyme (14.4 kDa) were used. A semi-dry blotting system was used for the electroblotting to transfer proteins from the SDS-PAGE gel onto a nitrocellulose membrane (Schleicher and Schuele). The probe, a nickel activated horseradish-peroxidase probe (INDIA™ HisProbe-HRP, Pierce, Rockford, USA) diluted 1:3000 in TBST buffer, was visualized by chemiluminescence. The positive control used was a six histidine tagged ureate oxidase (Pierce, Rockford, USA).

4.3.3 Development of an HPLC based aromatase assay in *P. pastoris*

P. pastoris strains GS115/pPIC3.5K-CYP19 and GS115/pPIC3.5K (control) were grown as described in section 4.3.2.4 and androstenedione metabolism was investigated. The parent vector strain (GS115/pPIC3.5K) was included to serve as a control. Actively growing yeast cells (5 x 10^7 cells/ml) were induced with methanol for 48 h in a total volume of 200 ml in a shaking incubator at 30°C. After 48 h induction, the cell suspension (4 ml per reaction) was harvested by centrifugation at 2000 x g for 5 min at room temperature. The cell pellets were washed with phosphate buffered saline (PBS) and resuspended in fresh BMMY (1 ml) induction media. The cell suspension was pre-incubated for 15 min at 30°C in an Erlenmeyer flask (25 ml), whereafter the reaction was initiated by adding an equal volume of androstenedione (10 µM) in BMMY media (1 ml).

The conversion reaction was assayed for 2 h by taking samples (50 µl) at 0, 10, 20, 30, 60 and 120 min. Steroids in the media were extracted with methylene chloride (100:1 solvent to incubation mixture). The water phase was aspirated and the remaining methylene chloride was evaporated under a stream of nitrogen gas. The dried steroids were redissolved in a mixture of ethanol:methanol:diisopropyl ether (12.5:12.5:75) (140 µl) for HPLC analyses. HPLC analyses was carried out on a Waters HPLC, equipped with a model 590 solvent delivery system (Waters, Milford, MA), a Waters M490 UV detector (Waters, Milford, MA), a Waters U6K injector (Waters, Milford, MA) and a silica column (2 micron pore size, 15 cm in length, with an internal diameter of 3.9 mm, Waters, Milford, MA). Solvents used for the separation of the steroids were filtered through a 0.45 µm pore size membrane (Millipore) and degassed by sonication. Androstenedione and estrone were injected as standards to determine the elution time of the steroids on the silica column. The separation of the steroids was developed with an isocratic solvent mixture of ethanol:methanol:diisopropyl ether (12.5:12.5:75) over a 10 min period at a flow rate of 1 ml/min. Data were collected by the Delta Chromatography Data system (version 5.0, DataWorX Pty. Ltd.) and integration of the resulting chromatograms was done with CHROMuLAN software[2].

4.3.4 Purification of the aromatase protein

4.3.4.1 Preparation of yeast cells for protein purification

Recombinant *P. pastoris* strains GS115/pPIC3.5K-CYP19 (200 ml) and GS115/pPIC3.5K (200 ml) were grown as described in section 4.3.2.4. After optimal expression was reached, cultures were transferred to sterile Falcon tubes (50 ml) and centrifuged in a bench top centrifuge at 2000 x g for 10 min at room temperature. The cell pellets were resuspended in buffer A1 (5 ml, 50 mM sodium phosphate, 1 mM PMSF, 20 % glycerol (v/v), pH 7.6) per 5 g wet weight / 5 ml and centrifuged at 2000 x g for 10 min at 4°C. Buffer A2 (cell lysis buffer, 1 ml, 50 mM sodium phosphate, 1 mM PMSF, 20 % glycerol (v/v), 1 mM EDTA, 10 mM β-mercaptoethanol, pH 7.6) per 0.5 g wet weight was added to all tubes. An equal volume of acid washed glass beads were

[2] http://www.chromulan.org

added and cells were disrupted by vortexing (10 x 30 s) at 4°C with intermediate cooling on ice. The disrupted cells were transferred to centrifuge tubes (50 ml) and centrifuged at 14000 x g for 30 min at 4°C. From the lysate (crude extract) 100 µl was collected for analysis with SDS-PAGE. Emulgen 913 was added drop wise to a final concentration of 1% (v/v) while stirring the crude extract at 4°C. This step was carried out for 4 h, whereafter 100 µl of the yeast microsomes were collected for later analysis. The microsomes were filtered through (0.45 µm, Millipore) and 100 µl of the resulting soluble membrane fraction was collected. The sample was purified on a HIS-Select™ cartridge (Sigma) by immobilized metal affinity chromatography (IMAC)[3].

4.3.4.2 IMAC purification of aromatase

IMAC was carried out on a ÄKTA*prime* system equipped with an internal UV detector and absorption was monitored at 280 nm. All buffers used for the purification of aromatase were filtered (0.45µm, Millipore). The column was equilibrated with binding buffer A (50 mM sodium phosphate, pH 8, 300 mM NaCl, 20 % glycerol (v/v), 0.1 % Emulgen 913 (v/v), 5 mM imidazole). The crude soluble fraction (20 ml) was applied manually onto the column. The His-tagged proteins were eluted with elution buffer B (20 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole, pH 7.4) at a flow rate of 1 ml/min. Fractions (1 ml) were collected with a fraction collector and the data collected with the P*rime*V*iew* (Amersham Biosciences) software. A peak was observed once the percentage concentration of elution buffer B reached 65 %. The fractions corresponding to the elution time of the peak were concentrated (Ultrafree – 4 centrifugal filter and tube, Millipore) to 50µl and prepared for SDS-PAGE and Western blot analyses as described in section 4.3.2.6. Samples (100 µl), collected during the preparation of the cells (see section 4.3.4.1), prior to IMAC purification were also included in these analyses as controls.

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4.4 Results

4.4.1 Transformation of *E.coli* JM109 cells – Verification of integrity

After the isolation of pGEM-T-CYP19 and pPIC3.5K plasmids, both were digested with NotI. The CYP19 gene was gel-purified after electrophoresis of the digested fragments. The digested plasmid was phosphorilated to minimize religation of the sticky ends. After ligation of CYP19 into pPIC3.5K and transformation of *E. coli* JM109 cells with the resultant construct (pPIC3.5K-CYP19), the plasmid was isolated from the transformants. The construct was subjected to DNA sequencing. PCR was carried out with both the sense (5'AOX1) and antisense (3'AOX1) primers. Two DNA bands were present in the PCR product of the construct (Fig. 4.4.1, lane 1). The larger fragment (~2200 bp) corresponded to the amplified *AOX*1 gene of the pPIC3.5K plasmid, while the smaller fragment (~1771 bp) corresponded to the amplified *CYP19* gene, which included the flanking 5' PAOX1, and 3' *AOX*1 regions.

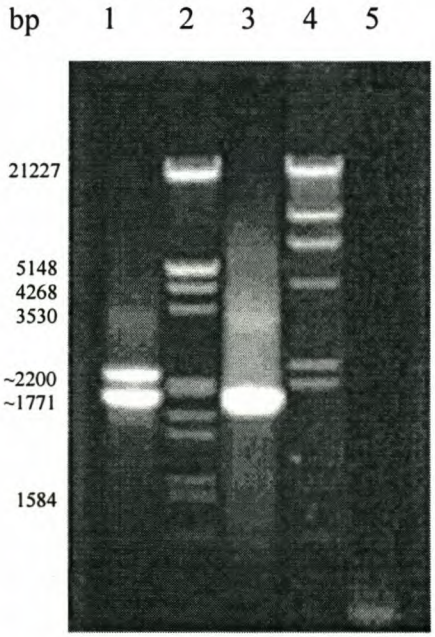

Fig. 4.4.1. Analysis of PCR amplification of pPIC3.5K-CYP19. Amplified products (20 µl) were electrophoresed on a 1 % agarose gel, stained with EtBr and visualised on a UV transilluminator. Two clear fragments were visible, the larger fragment corresponding to the amplified AOX1 gene of the pPIC3.5K plasmid and the smaller fragment corresponded to the amplified CYP19 gene including the flanking 5'PAOX1 and 3'AOX1 regions. Lane 1: pPIC3.5K-CYP19 amplified with both a sense and antisense primer. **Lane 2:** λ-marker digested with both EcoRI and HINDIII (Promega). **Lane 3:** Positive control (pPIC3.5K). **Lane 4:** λ-marker digested with HINDIII (Promega). **Lane 5:** Negative control (no DNA).

4.4.2 Transformation of *P. pastoris* with CYP19 and verification of integrity into *P. pastoris* GS115

Spheroplasting did not provide high transformation efficiency. Electroporation was more efficient for the transformation of *P. pastoris,* hence this method was the method of choice for all subsequent transformations.

PCR amplification followed by 1 % agarose gel electrophoretic analysis of lysed His^+ transformants showed that the CYP19 was integrated correctly into the *P. pastoris* genome. Two bands were visible after electrophoresis. The larger fragment (~2200 bp) corresponded to the amplified *AOX*1 gene of the pPIC3.5K plasmid, while the smaller fragment (~1771 bp) corresponded to the amplified *CYP19* gene with the flanking 5' P*AOX*1 and 3' *AOX*1 regions (Fig. 4.4.2, lane 4). *P. pastoris* cells with only the parent vector pPIC3.5K present produced two PCR products – 492 bp and 2100 bp (Fig. 4.4.2, lane 2).

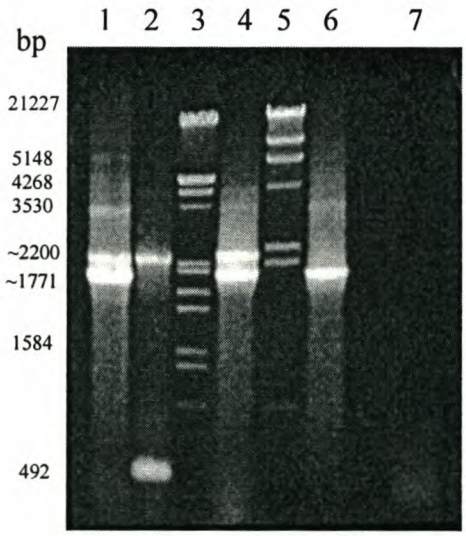

Fig. 4.4.2. Analysis of the integration of CYP19 into the yeast genome. P. pastoris clones were subjected to direct PCR screening and amplified products (20 µl) were electrophoresed on a 1 % agarose gel, stained with EtBr and visualised on a UV transilluminator. Two clear fragments were visible, the larger fragment corresponding to the amplified AOX1 gene of the pPIC3.5K plasmid and the smaller fragment corresponded to the amplified CYP19 gene including the flanking 5'PAOX1 and 3'AOX1 regions. Lane 1: GS115/pPIC3.5K-CYP17. **Lane 2:** GS115/pPIC3.5K with no insert. **Lane 3:** λ-marker digested with EcoRI and HindIII. **Lane 4:** GS115/pPIC3.5K-CYP19 amplification resulting in 2 bands (2200 and 1771 bp). **Lane 5:** λ-marker digested with HindIII (Promega). **Lane 6:** Positive control containing CYP19 fragment (1771 bp) amplified from pPIC3.5K-CYP19. **Lane 7:** Negative control, PCR amplification without DNA.

4.4.3 Expression of aromatase in *P. pastoris*

After the successful transformation of *P. pastoris*, GS115/pPIC3.5K-CYP19 cells were induced to express human aromatase as described in section 4.3.2.4. A time course study was done with the induced cells over a period of 3 days to determine the optimum time of expression. The optimum expression time was 48 h. Fig. 4.4.3 (A) illustrates the Western blot analysis of the expression of human aromatase induced by methanol over a period of time. The Western blot analysis, illustrated in Fig. 4.4.3 (B), represents different positive clones of GS115/pPIC3.5K-CYP19 at different expression times after induction by methanol. Human His-aromatase in Western blot analysis was detected with a His-tag probe directed against histidine-rich proteins at a dilution of 1:3000. Carbonic anhydrase of the Rainbow marker is also detected with the His-tag probe, since it is a histidine-rich protein.

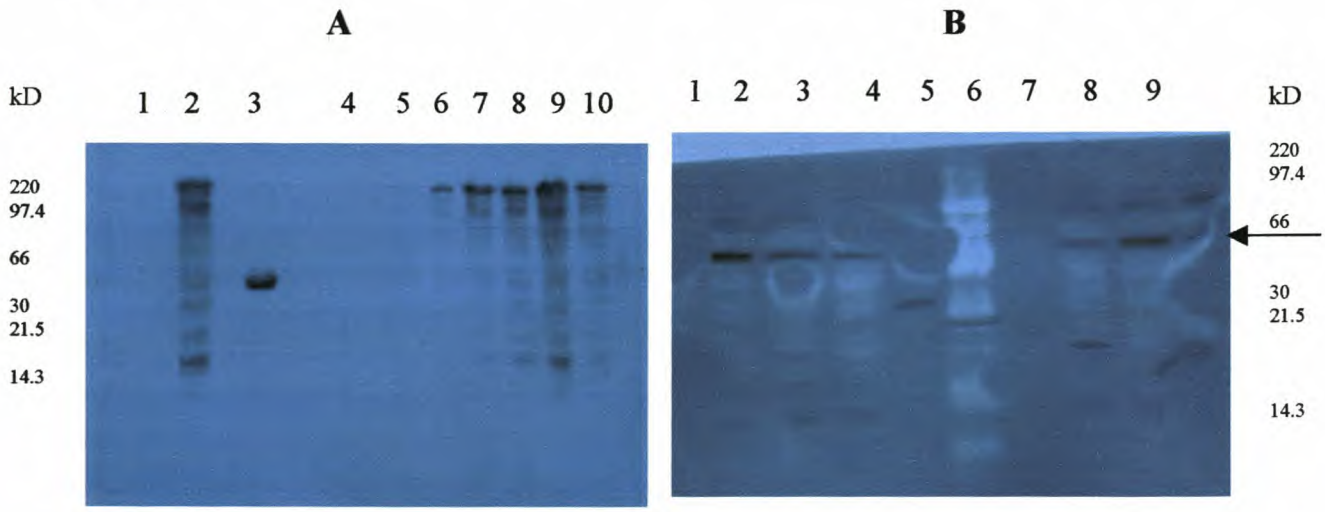

Fig. 4.4.3. (A) Western blot analysis of total intracellular yeast proteins of GS115 cells induced to express aromatase for 3 days Lane 1 and 2: GS115 cells transformed with pPIC3.5K vector at 0 and 72 h, respectively. **Lane 3:** Rainbow marker. **Lanes 4-10:** GS115 cells transformed with pPIC3.5K-CYP19 vector at 0, 6, 12, 24, 36, 48 and 60 h, respectively. Detection was carried out using an INDIA HisProbe-HRP at 1:3000. **(B) Western blot analysis of total intracellular proteins of specific clones of GS115 cells induced to express aromatase for 2.5 days. Lane 1:** Parent vector, 0 h; **Lane 2:** CYP17, 48 h; **Lane 3:** CYP19, Clone 2, 48h; **Lane 4:** CYP19 Clone 15, 48 h; **Lane 5:** Positive control; **Lane 6:** Rainbow marker; **Lane 7:** Negative control; **Lane 8:** CYP19 Clone 1, 60 h; **Lane 9:** CYP17, 60 h. Detection was carried out using an INDIA HisProbe-HRP at a 1:3000 dilution. The arrow indicates aromatase at ± 55 kD.

Fractions were loaded on a maxi-SDS-PAGE gel (10 %) and subjected to Western blot analyses to obtain better separation of the proteins and to further confirm the expression of aromatase. Fig. 4.4.4 (A) is the stained SDS-PAGE gel of the total intracellular yeast

proteins of *P. pastoris* GS115 cells induced to express aromatase. GS115/pPIC3.5K-CYP17 was included as a positive control. The Western blot analysis (Fig. 4.4.4 (B)) was carried out as previously described.

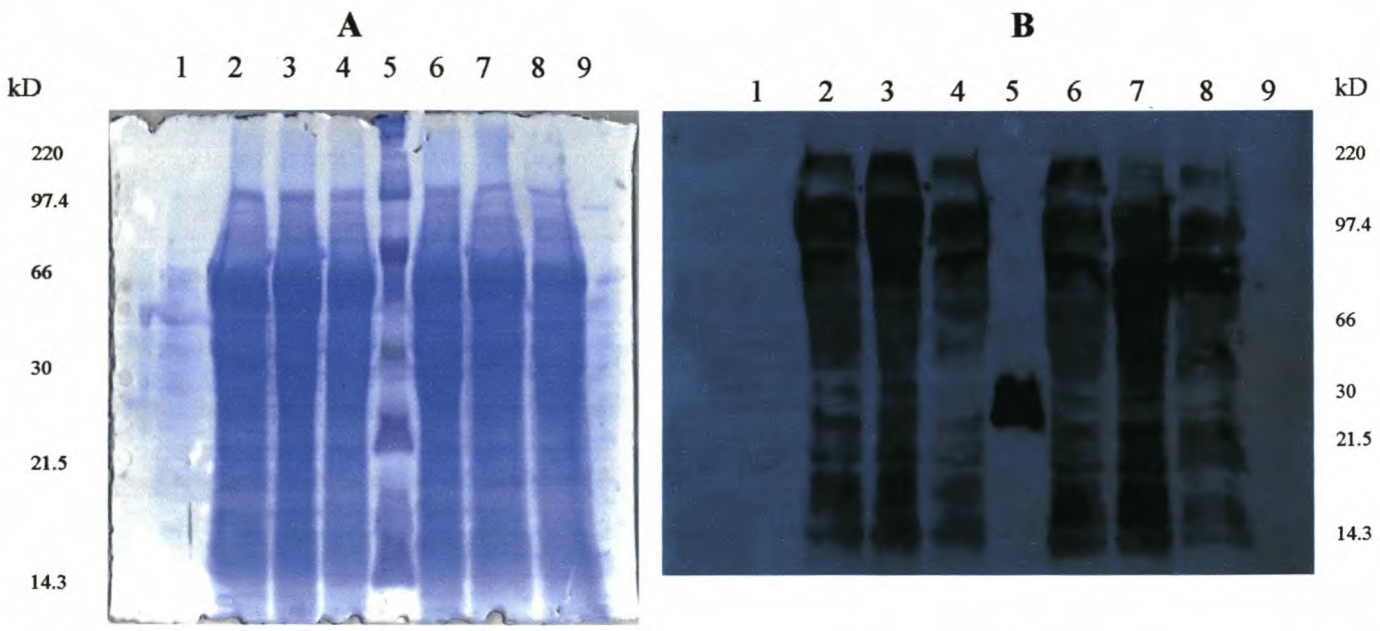

Fig. 4.4.4. Analysis of total GS115 intracellular proteins at 48 h after induction to express aromatase. (A) Image of SDS-PAGE (10%) and (B) Western blot analysis using a His-tagged probe at 1:3000. Lane 1: CYP19 clone 1, 0 h; **Lane 2:** CYP19 clone 1, 24 h; **Lane 3**: CYP19 clone 2, 24 h; **Lane 4**: CYP19 clone 1, 48 h; **Lane 5:** Rainbow marker; **Lane 6:** CYP19 clone 2, 48 h; **Lane 7:** CYP17, 48 h; **Lane 8:** Parent vector, 48 h. **Lane 9:** Negative control.

4.4.4 Conversion of androstenedione to estrone by aromatase

Since a His-tag probe directed at histidine-rich proteins was used for the identification of aromatase, it was not possible to assume from the Western blot analyses alone that human aromatase was being expressed in the yeast cells. A conversion assay was therefore carried out to confirm that aromatase was indeed being expressed as an active enzyme in the yeast cells. Human aromatase activity was determined in the yeast cells, by assaying the conversion of androstenedione (10 μM) to estrone. An HPLC based aromatase assay was developed for the detection and separation of androstenedione and estrone. The separation was developed isocratically over 10 min with ethanol:methanol:diisopropyl ether (12.5:12.5:75) as solvent. Fig. 4.4.5 (I) shows the separation of the standards, androstenedione (2 mg/ml) and estrone (4 mg/ml) while Fig.

4.4.5 (II) represents a typical separation of the steroids obtained from the expression of human aromatase in *P. pastoris* after a 2 h assaying period.

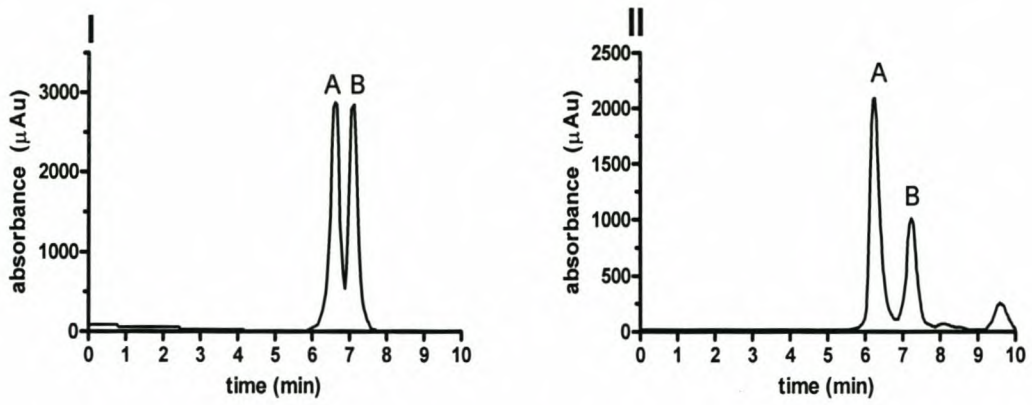

Fig. 4.4.5. (I) Elution profile of the standards for the conversion of androstenedione to estrone. Estrone (A) elutes at 6.63 min and androstenedione (B) at 7.11 min. and (II) Elution profile of the conversion of androstenedione (B) to estrone (A) by the transformed yeast cells after 2 h of assaying.

Samples were collected, in duplicate, at specific time intervals and the conversion assay was repeated four times. Data was collected by a Delta Chromatography Data System version 5.0 (DataWorX Pty.Ltd.) and analysed using CHROMuLAN[4]. Fig. 4.4.6 summarizes the aromatase conversion in terms of substrate (androstenedione) depletion and the product (estrone) formation. After 2 h, approximately 30 % of the androstenedione was converted to estrone. Also included in the figure is the conversion result of the yeast cells transformed with the vector with no insert. The rate of androstenedione conversion by aromatase in the yeast cells was significantly lower than the rate of progesterone conversion by yeast cells transformed with human CYP17, which showed a conversion of approximately 80 % progesterone after 1 h (see section 6.3.1).

[4] http://www.chromulan.org

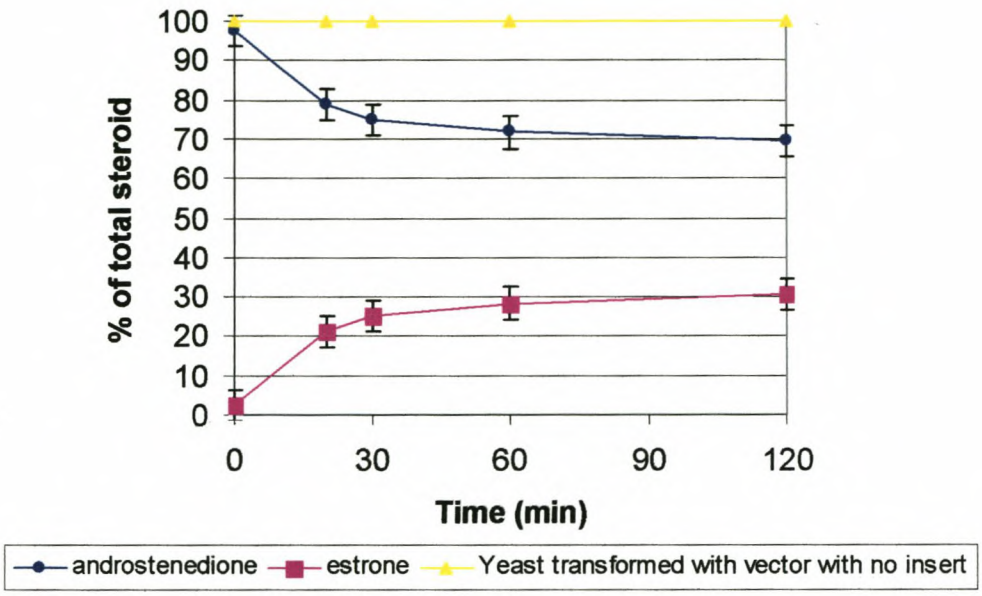

Fig. 4.4.6. Conversion of androstenedione to estrone by the human aromatase enzyme in *P. pastoris*. After 2 h, 30 % of the substrate is converted to product.

4.4.5 Purification of the expressed aromatase protein

Once the *P. pastoris* cells were expressed and analysed for aromatase expression the protein was isolated and subsequently purified by IMAC.

Fig. 4.4.7 shows the elution profile of the His-tagged aromatase during IMAC purification. Four fractions, corresponding to the visible peak, were super-concentrated to 50 µl and prepared for SDS-PAGE (Coomassie staining) and Western blot analysis (Fig. 4.4.8).

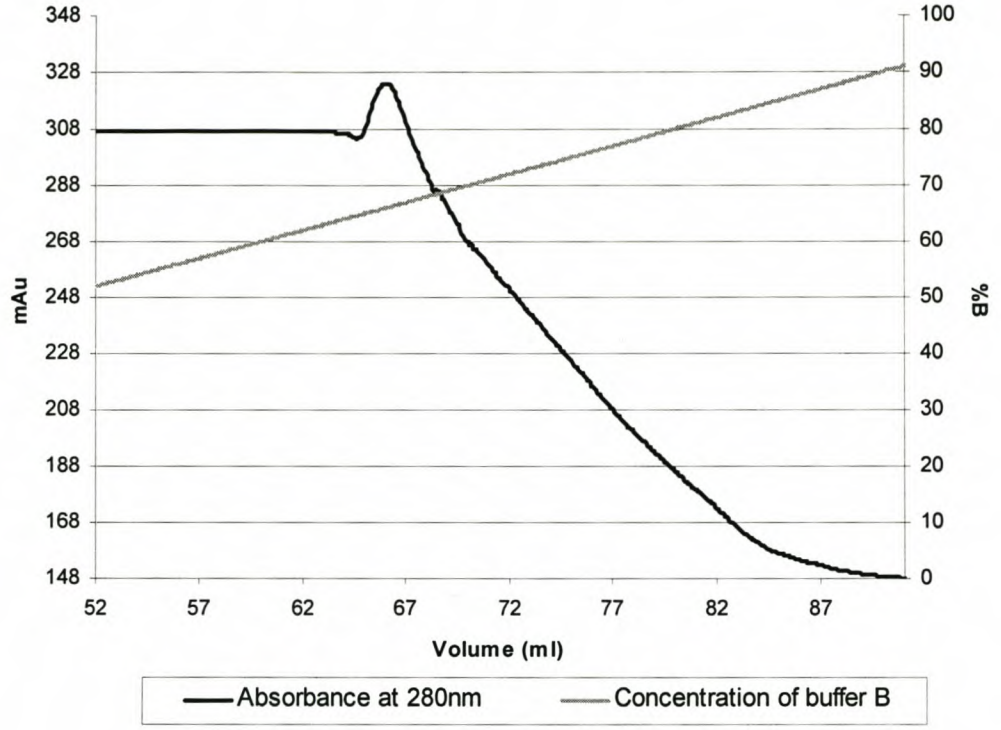

Fig. 4.4.7. Elution profile of the purified His-tagged protein. The dark line represents the UV absorbance. The protein eluted at 65 % buffer B.

The purified aromatase samples were electrophoresed on a 10 % denaturing polyacrylamide gel and visualized after staining (Fig. 4.4.8 (A)). Identical samples were electrophoresed and transferred to a nitrocellulose membrane for Western Blot analysis (Fig. 4.4.8 (B)). The samples that were collected during the preparation of yeast microsomes for the isolation of the aromatase protein were included in the SDS-PAGE and Western blot analysis. Only the concentrated purified fraction (Fig. 4.4.8 (B), lane 5) was, however, detected with immunoblotting using the His-probe, indicating a low level of expression of the aromatase protein in the yeast. The lighter bands (Fig. 4.4.8 (B), lane 5) may be due to the degradation of the aromatase protein.

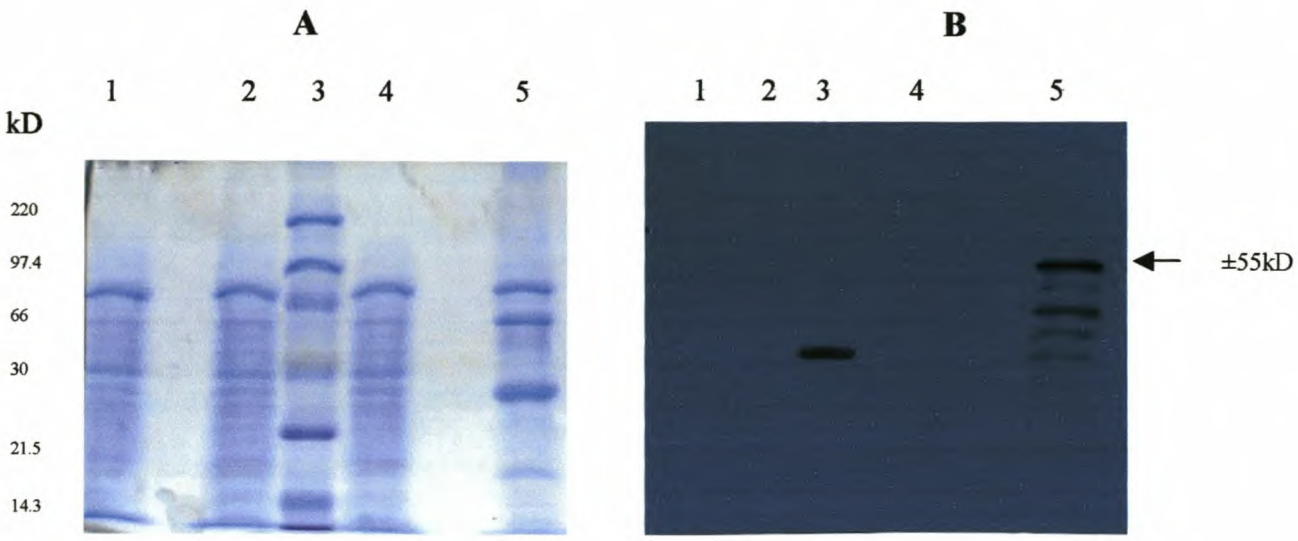

Fig. 4.4.8. Analysis of the purification of aromatase from *P. pastoris*. (A) Image of SDS-PAGE (10%) and (B) Western blot analysis using a His-tagged probe at 1:3000. Lane 1: Crude extract. **Lane 2:** Microsomal fraction. **Lane 3:** Rainbow marker. **Lane 4:** Filtered microsomal fraction that was loaded onto the column. **Lane 5:** Concentrated purified fraction obtained from the IMAC purification. A clear dark band of the expressed aromatase protein (55 kD) can be seen in (B).

4.5 Discussion

Cytochrome $P450_{arom}$, a membrane bound, microsomal enzyme, displays tissue-specific regulation. The aromatase protein is expressed in the ovaries, placenta, brain and adipose tissue of mammals and plays a crucial role in steroidogenesis. It catalyses three sequential oxygenation reactions that converts C19 androgens to aromatic C18 estrogens [6,8,9]. Estrogen biosynthesis by adipose tissue increases with body weight and age, and this possibly leads to increased incidence of both breast cancer and EC, which is believed to develop and progress as a result of abnormal expression of aromatase [10,11,12].

The study of human cytochromes P450 is complicated by the relative low expression levels in tissues and organs where these hemoproteins are active. Heterologous expression of these proteins can facilitate investigations of enzyme activities and structure/function relationships. In addition, heterologous expression can also aid in the evaluation of the effect of xenobiotics on these enzymes as well as provide sufficient pure cytochrome P450 proteins for the production of antibodies for immunochemical and

immunohistochemical studies. These studies could include the localization of aromatase in breast tissue.

P. pastoris, an eukaryotic methylotrophic yeast, was chosen as the heterologous expression system in this study since it provides all the advantages of higher eukaryotic expression, such as protein processing and folding, and posttranslational modification [72-75].

The successful integration of the *CYP19* gene into the *P. pastoris* genome, was achieved by electroporation, verified with direct PCR screening of transformed cells and analysed by agarose gel electrophoretic analysis (Fig. 4.4.2).

SDS-PAGE and Western blot analyses of the cells pellets of *P. pastoris* GS115 cells expressing aromatase, identified a protein fraction corresponding to a molecular mass of 55 kD. The concentration of this protein, according to the intensity of the chemiluminescent signal, gradually decreased from 48 h to 72 h. A possible reason for the relative low expression of the CYP19 could be the retention of expressed aromatase in the ER of the yeast cell or a low copy number integration of CYP19 into the yeast genome. It must however, be noted that high plasmid copy number is not always a guarantee for increased protein production in *P. pastoris* [74,104].

The recombinant *P. pastoris* cells converted androstenedione to estrone indicating a functional expressed enzyme in *P. pastoris* with the endogenous yeast reductase system capable of electron transport to the human aromatase. It is interesting to note that after 2 h the conversion of androstenedione to estrone reached equilibrium. This incomplete conversion of substrate can be ascribed to two factors: (a) the supply of dissolved oxygen in the small 25 ml Erlenmeyer flasks used for incubation could be insufficient to sustain a high rate of substrate conversion or (b) the reducing equivalents available could not support a higher substrate conversion rate. The influence of these two parameters on substrate conversion by aromatase will be investigated in follow up studies in this laboratory, but is beyond the scope of this investigation.

For the isolation of aromatase from *P. pastoris* cells, it was possible to solubilise aromatase from the membrane fraction with the addition of Emulgen 913 to the cell lysate. IMAC was used to successfully purify the recombinant human aromatase from *P.*

pastoris. Inclusion of β-mercaptoethanol in the solubilisation buffer prevented co-aggregation of aromatase and endogenous yeast proteins thereby facilitating the elution of unwanted proteins during the purification process. An intense signal was detected with the His-tag probe at a molecular mass corresponding to 55 kD (Fig. 4.4.8 (B)). The less intense bands at lower molecular masses could be attributed to the degradation of the aromatase protein.

An increase in yield of aromatase can potentially be accomplished by producing multi-copy strains with the pPIC3.5K-CYP19 by following different transformation strategies, such as truncating the amino terminal end, although it may not produce a functional protein.

The results obtained in this study indicate that *P. pastoris* is a suitable heterologous expression system for the production of unmodified human aromatase. The enzyme was active *in vivo* and could also be isolated from cell lysate using IMAC. With modifications to the expression vector to yield a higher copy number, sufficient amounts of aromatase protein can be produced for further enzymatic studies as well as the production of polyclonal and monoclonal antibodies.

CHAPTER FIVE

Cytochrome P450 17α-hydroxylase/17,20-lyase

5.1 Introduction

The cytochrome P450 17α-hydroxylase/17,20-lyase represents a major metabolic branch point in adrenal steroidogenesis between glucocorticoid and mineralocorticoid production as well as between glucocorticoid and androgen production [10,14,15,79]. P450c17 has dual activity. It catalyses the hydroxylation, at C17, of the C_{21} steroids and the C17, C20-lyase of C17 hydroxylated steroids. The ratio of the two catalytic activities of P450c17 is influenced by the tissue in which it is expressed, since different tissues exhibit different steroidogenic requirements [2,5,7,79-83]. P450c17 is expressed as a membrane bound protein in the ER of the adrenals, ovaries and testis as well as various non-steroidal tissues, which include the brain, liver and intestines [5,14,15,19,79,81,83]. This hemoprotein contains two transmembrane segments at the NH_2-terminus that anchor the protein to the membrane through the formation of a hairpin loop structure. When P450c17 is analyzed by SDS-PAGE, it exhibits a single band corresponding to a molecular mass of approximately 53 kD [5,14,15,19,79,81,83]. The 17α-hydroxylase function of P450c17 is responsible for converting pregnenolone and progesterone to 17preg and 17OHprog, respectively. The second activity of this enzyme, namely the 17,20-lyase function, cleaves 17OHpreg and 17OHprog at their steroid side chain to yield DHEA and androstenedione respectively (Fig. 5.1.1) [2,5,7,10,14,15,19,79-83].

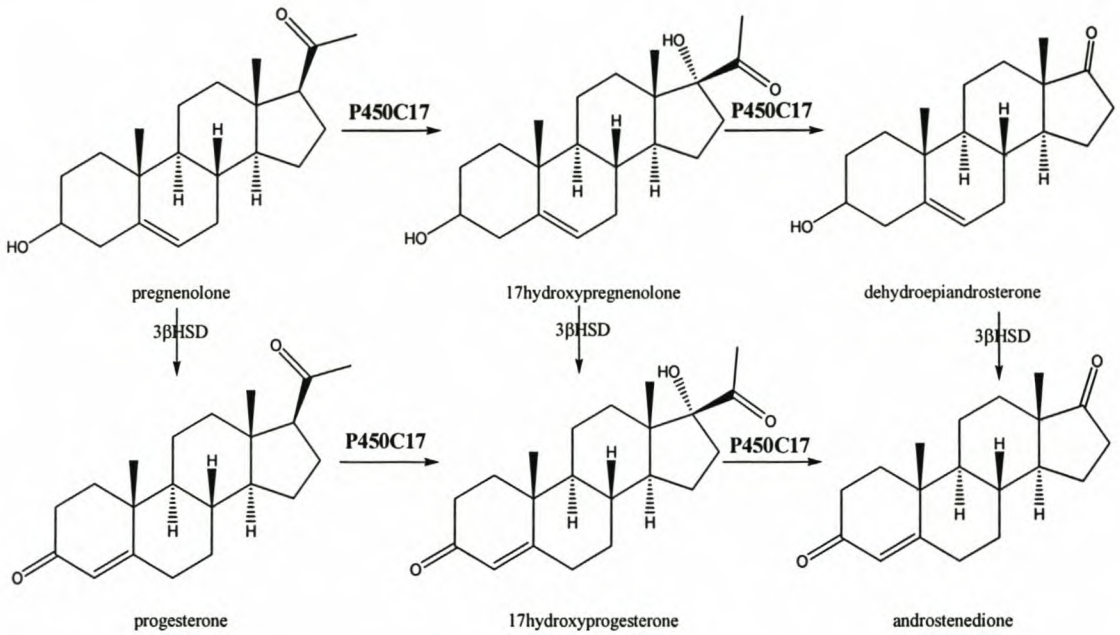

Fig. 5.1.1. Reactions catalysed by P450c17. 3βHSD; 3β-hydroxy-Δ^5-steroiddehydrogenase-isomerase.

5.2 The CYP17 gene

The cytochrome P450 designated *CYP17* (Fig. 5.2.1) is located on chromosome 10 of the human genome. It is a single copy gene, mapped to q24.3 of chromosome 10. The gene spans 6569 bp on the chromosome and is divided into eight exons which span 6 kb and seven introns. The full-length cDNA of P450c17 is 1527 bp in length and encodes for a single 508 amino acids polypeptide protein [7,82,84,85]. Adrenocorticotropin (ACTH) regulates the biosynthesis of all major adrenocortical hormones. This is achieved by maintaining transcriptional pressure on the genes encoding the biosynthesis of these hormones *via* the second messenger cAMP. Each gene uses its own unique cAMP response system during this coordinate response. A number of consensus sequences are found in the 5' flanking region of the *CYP17* gene. These sequences, the *cis*-acting cAMP regulatory elements (CRE) and activator protein 1 (AR-1) or activator protein 2 (AR-2) binding sites bind cAMP to induce gene transcription. For the *CYP17* gene to be expressed, only 310 bp of the exon is required [86-88]. In the past it was thought that families of genes or at least subfamilies are regulated in the same manner. However, this assumption is incorrect. A combination of transcriptional activation, posttranscriptional

and posttranslational regulation regulates the genes encoding the cytochrome P450-dependent enzymes [7,85-88].

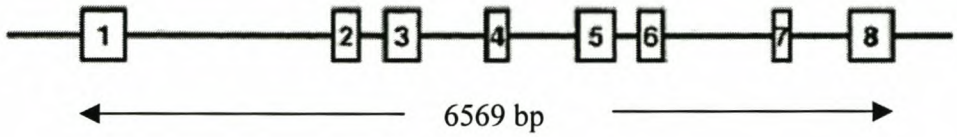

Fig. 5.2.1. Schematic representation of the human *CYP17* gene, indicating the approximate locations and sizes of the 8 exons.

5.3 The mechanism of CYP17 catalysis

The P450c17 enzyme is responsible for three different reactions in humans: firstly, the 17α-hydroxylation of pregnenolone and progesterone to form 17OHpreg and 17OHprog respectively; secondly, the 17, 20 carbon-carbon bond cleavage of 17OHpreg and 17OHprog. A third reaction, the 16α-hydroxylation of progesterone, is found only in humans. The ratio of 16OHprog to 17OHprog for the human P450c17 catalysed reaction is approximately 1:4 [5,10,14,15,79-84,89-92].

The hydroxylation of progesterone at the C17 position is well documented. The activated oxygen molecule, known as oxene, is formed by the heterolytic cleavage of the iron-bound peroxy intermediate. A substrate-based radical and an equivalent of an iron-bound hydroxyl radical are formed because the hydrogen atom is abstracted from the substrate by the oxene. The recombining of these two radical species produces the hydroxylated product [89]. Fig. 5.3.1 is a schematic representation of the 17α-hydroxylation reaction cycle of P450c17 [89].

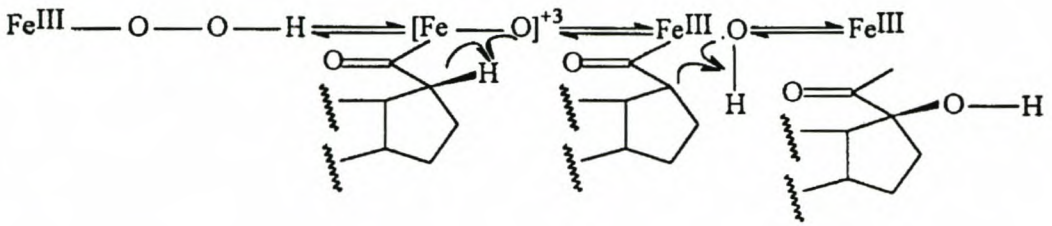

Fig. 5.3.1. Schematic representation of the 17α-hydroxylation reaction cycle catalyzed by P450c17 that occurs *via* oxene chemistry [89].

The P450c17 enzyme also catalyses a carbon-carbon bond cleavage reaction, which is not as well understood as the 17α-hydroxylation reaction. It has been reported that it is possible that a substrate-bound ferric peroxy intermediate is involved in P450c17 oxidations [89,90]. P450c17 catalyses the formation of androstenedione from 17OHprog *via* a ferric-peroxy-dependent pathway also known as a Baeyer-Villiger rearrangement. The intermediate that is formed during this conversion could possibly rearrange heterolytically *via* this Baeyer-Villiger rearrangement to form a hemiacetal or rearrange homolytically to form the gem diol [89,90]. Fig. 5.3.2 is a schematic representation of the peroxide chemistry reaction, catalyzed by P450c17 [89]. The intermediates are hydrolyzed to yield a C19 steroid. It is also possible that protons play a role in the differentiation between oxene and peroxide chemistry. The chemistry and product identity of the P450c17 reaction is determined by the state of protonation of the ferric peroxide intermediate [89,90].

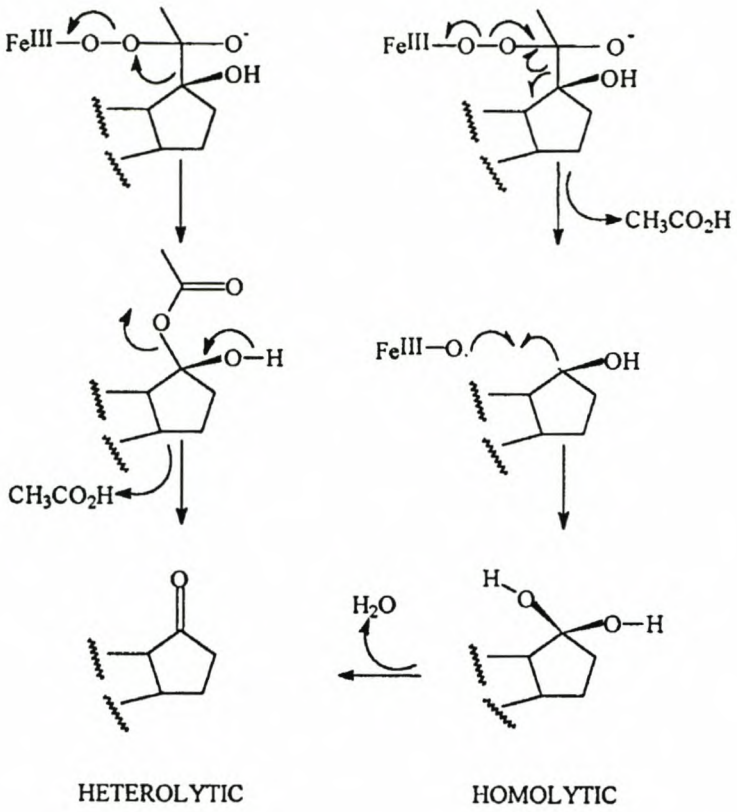

Fig. 5.3.2. Schematic representation of peroxide chemistry as catalyzed by P450c17. The intermediate could rearrange homolytically or heterolytically *via* the Baeyer-Villiger rearrangement [89].

5.4 The role of CYP17 in steroidogenesis

In the human adrenal gland, there are four main enzymes involved in steroidogenesis. In the mitochondria $P450_{scc}$ and P450c11 are found, while P450c21 and P450c17 are located in the ER [2,10,14,15,79]. The three different steroid hormones that are synthesized in the adrenal glands are mineralocorticoids, glucocorticoids and androgens. The mineralocorticoids are involved in regulating electrolyte and water balance, while the glucocorticoids regulate carbohydrate, protein and lipid metabolism. The androgens play an essential role in secondary sex characteristics [7,15,93].

As mentioned before, P450c17 has dual activity and is expressed in the *zonae fasiculata* and *reticularis* of the adrenal ER. In these two *zonae*, pregnenolone acts as substrate to produce glucocorticoids. In the *zona glomerulosa,* the cAMP concentration is low and

therefore no P450c17 is expressed since cAMP is crucial for inducing gene expression as mentioned in section 5.1 [14,15,80,87,93-95]. Fig. 5.4.1 is a schematic representation of a cross-section of the adrenal gland, showing the different zones[5].

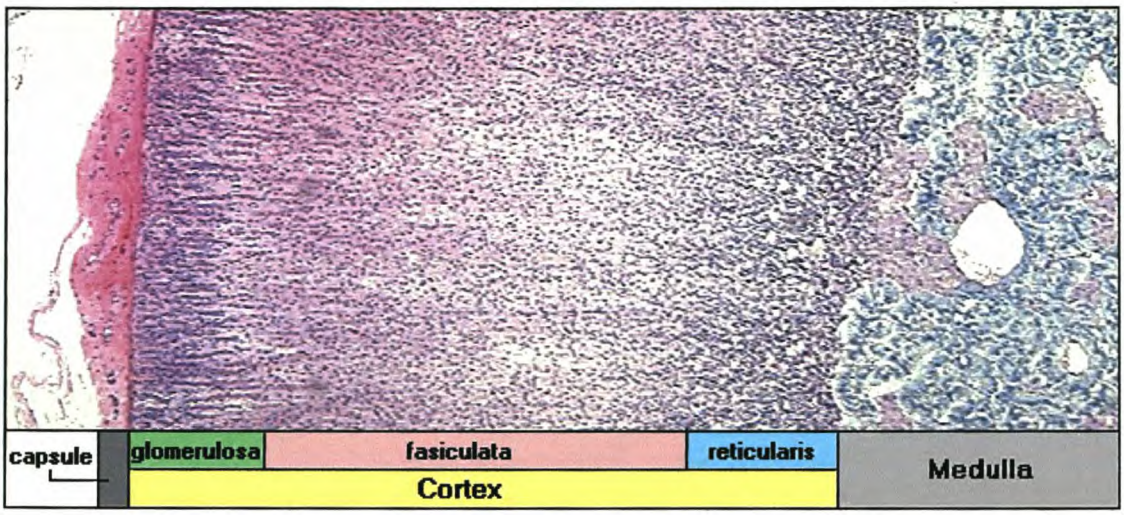

Fig. 5.4.1. Schematic representation of a cross section of the adrenal gland. It shows the different zones of the gland. P450c17 is expressed in two zones in the cortex, namely the *zonae fasiculata and reticularis*.

While the *theca interna* cells in the ovaries express P450c17 there is very low expression in the granulosa cells. Androgens are produced in the *theca interna* cells *via* P450c17, whereafter these androgen precursors can move to the granulosa cells where estrogens are produced [80,83,95,70].

When the P450c17 enzyme cannot catalyze the 17α-hydroxylase or 17, 20-lyase reactions, no sex hormones are produced. This deficiency in the enzyme leads to the production of female genitalia despite of the genetic sex. If only the 17, 20-lyase activity is lost, P450c17 can still catalyze the hydroxylation of the C_{21} steroids, which means that sufficient cortisol is still being produced. However, production of sex hormones is still lost [10,80,83,85,96].

[5] http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/adrenal/histo_overview.html

5.4.1 P450c17 substrates

The mitochondria produce pregnenolone and this steroid subsequently moves to the ER. In the ER, either P450c17 or 3β-HSD can convert it to the steroid intermediates. When P450c17 hydroxylates pregnenolone at the C17 position, it yields 17OHpreg. The lyase activity of P450c17 can then convert 17OHpreg to DHEA. 3β-HSD can convert pregnenolone or 17OHpreg to yield progesterone or 17OHprog respectively, providing substrates for P450c17. 3β-HSD also converts DHEA to androstenedione. P450c17 hydroxylates progesterone to form 17OHprog and the C17-C20 carbon-carbon bond of 17OHprog is cleaved to yield androstenedione. In the human, progesterone is also 16α-hydroxylated to yield 16OHprog [7,10,14,15,79-84,89-95,96,97].

The dual activity of P450c17 is differentially regulated and responsible for the important shift in steroid metabolism in different tissues. Factors that are thought to influence the ratio of these two reactions include the electron flow to the enzyme as well as the enzyme, cytochrome b_5 [19,83].

5.5 CYP17 and cancer

In the 5'-untranslated region of *CYP17*, a single base pair polymorphism (T to C substitution) creates an Sp1-type (CCACC box) promoter site that is 34 bp upstream from the initiation site of translation but 27 bp downstream from the transcription start site. Due to this base pair change, a recognition site is created for the MspAI restriction enzyme. Researchers have used *Msp*AI digestion of a PCR fragment to designate two alleles. The undigested allele is called A1 and the digested allele A2 [84,85,92]. This polymorphism of the *CYP17* gene has been implicated, in many studies, to be involved with the onset of EC, breast cancer, polycystic ovarian syndrome (PCOS) and higher levels of serum estradiol in women [63,84,85,91,92,96,97]. In men, it has been associated with increased risk of prostate cancer, benign prostatic hyperplasia (BPH) and male-pattern baldness [85]. However, researchers are not sure whether the CCACC site affects endocrine status, whether the A1/A2 polymorphic site is a causative mutation or if it is simply a marker for another mutation affecting enzymatic activity. An additional Sp-1 type promoter site in the 5'-untranslated region of CYP17 is associated with the A2 allele. It is thought that the number of 5' promoter elements correlates with promoter

activity. It is speculated that this CCACC site enhances CYP17 transcription that leads to increased activity and ultimately increased serum estrogen levels [63,84,85,91,92,96,97].

As discussed in section 3.4, four steroids play an important role in tumor endocrine response. Since progesterone and estrogen have an interrelationship, progesterone has also been studied for its effect on breast cancer. Huggins *et al.* [52] have suggested that progesterone plays an important role in tumor growth stimulation. Progesterone may control breast tumor growth by directly affecting the tumor or by modifying actions of other steroid hormones which influence the mammary gland. McGuire *et al.* [48] found that estradiol enhances the sensitivity of the tissue to progesterone through increased progesterone receptor levels.

The initiation or promotion of EC is associated with excess estrogen and androgen levels as well as with hyperinsulinemia. It is possible that some allelic polymorphisms of the genes involved in steroidogenesis contribute to EC susceptibility. Although mutations of so-called 'normal' genetic variability are usually accompanied by visible changes, the influence of allelic polymorphisms on the phenotype is much less distinctive [63,91,92,96,97]. Bernstein *et al.* [96] found that the A2/A2 genotype of CYP17 is a risk in breast cancer but is under-represented in EC patients. They concluded that the A1/A1 genotype, which was thought to be the normal genotype, might be one of the risk factors for EC. In another study by Bernstein *et al.* [96], they compared CYP17 and CYP19 genetic polymorphisms. These studies showed that females with the highest risk of developing EC are carriers of the A1/A1 or A1/A2 genotype of CYP17 and the A6/A6 or A6/A7 CYP19 genotypes. These patients also exhibited increased aromatase activity in their tumors. Aromatase expression was only observed during EC, while no aromatase is expressed in the normal endometrium. Bernstein *et al.* [96] therefore suggested that a 'high risk' CYP19 or CYP17 genotype might transform into a 'high activity' phenotype, leading to increased estrogen production, hence supporting further tumor growth. Since aromatase is responsible for estrogen production this argument seems logical for CYP19 polymorphisms. For CYP17 there may be two explanations. Firstly, P450c17 catalyses androgen biosynthesis, the precursors for estrogen production. Secondly, it may be true

that hyperinsulinemia is involved by stimulating ovarian steroidogenesis and aromatase activity directly in endometrium cells [63].

Haiman *et al.* [91] found, in correspondence with the observations by Bernstein *et al.* [63,96], that women carrying the A2 allele of CYP17 are at decreased risk of developing EC. In postmenopausal women, they did not detect a definite association between the *CYP17* genotype and circulating steroid hormone levels. However, in premenopausal women with the A2 allele, the *CYP17* genotype and circulating steroid hormone levels may lead to increased production of all steroid hormones because of enhanced steroidogenesis. Although they found only modest associations between *CYP17* genotype and hormone levels, a strong association was observed between *CYP17* genotype and EC risk. This observation might suggest that the *A1* or *A2* alleles may be serving as markers of a functional variant in a nearby gene. Haiman *et al.* [91] and Hsieh *et al.* [97] found evidence that the A2 allele of *CYP17* is associated with a decreased risk for developing EC; however, the *CYP17* genotype has a negligible if any effect on steroidogenesis in post-menopausal women. In EC patients, a higher percentage of the A1/A1 genotype is present. There is still much controversy on whether the two alleles of *CYP17* are indeed involved in cancer risk, possibly due to multiple enzymatic processes and interactions [91,97]. Hsieh *et al.* [97] suggested that this polymorphism of the *CYP17* gene may be in linkage disequilibrium with an unidentified functional polymorphism in the *CYP17* gene which may influence EC risk.

It is controversial whether the variant allele of CYP17 is associated with the subset of breast cancer or PCOS. However, Miyoshi *et al.* [92] detected *CYP17* mRNA in breast cancer tumor tissue and found that it is significantly up regulated compared to normal breast tissue. These researchers suggested that the *CYP17* mRNA is involved in the initiation and development of breast cancer by increasing *in situ* synthesis of estrogens. Their results showed that the CYP17 genetic polymorphism is associated with intratumoral estradiol levels but not with *CYP17* mRNA expression levels [92].

As discussed earlier in section 3.4, there is ample evidence to support the link of genetic susceptibility to breast cancer through estradiol biosynthesis. Feigelson *et al.* [84] investigated the role of the CYP17 polymorphism in breast cancer risk. They found that

women carrying at least one A2 allele are at increased risk of advanced breast cancer. The A1/A1 genotype is associated with a later onset of menarche, suggesting that in females the CYP17 genotype may be a biomarker for the onset of ovulation and advanced breast cancer risk. However, they also observed that the later onset of menarche is only protective in women with the A1/A1 genotype [84]. Since the A2 allele may be responsible for an increased rate of transcription of CYP17 which will ultimately lead to increased estrogen production, it is possible that the protective benefit of late menarche is overridden by an increased lifetime exposure to estrogens. Feigelson *et al.* [84] concluded that women with the A2 allele might be good candidates for early intervention or possibly chemoprevention efforts.

Habuchi *et al.* [85] investigated the association between the CYP17 polymorphism and the risk of prostate cancer or BPH. They found that the A1/A1 genotype in men led to an increased risk of developing prostate cancer and BPH compared to the A2/A2 genotype. The heterozygote genotype is at moderate risk. As mentioned before, CYP17 is involved in the production of androgens as well as estrogens and while prostate cancers are androgen dependent, breast cancers are estrogen dependent. Habuchi *et al.* [85] concluded that the A1 allele definitely has a more androgenic effect on men, while the A2 allele has an estrogenic effect on women.

5.6 Summary

P450c17 is the product of the *CYP17* gene and represents a major branch point in steroidogenesis. The P450c17 enzyme is responsible for 2 different reactions, a hydroxylation reaction of pregnenolone and progesterone and a 17, 20-lyase reaction converting 17OHpreg and 17OHprog to yield DHEA and androstenedione respectively. P450c17 is a membrane bound enzyme found in the ER. The 17α-hydroxylation reaction occurs *via* oxene chemistry while the 17, 20-lyase reaction occurs *via* peroxide chemistry (the intermediate from the lyase reaction can rearrange either heterolytically or homolytically). P450c17 is expressed in the gonads and the adrenal cortex. In the *zonae fasiculata* and *reticularis* of the adrenal, pregnenolone metabolism yields glucocorticoids. In the *zona glomerulosa* there is low cAMP concentration and no P450c17 is expressed

since cAMP is a crucial regulator for the expression of this key enzyme. CYP17 is closely associated with the production of estrogens *via* CYP19. As a result, this enzyme has been studied extensively for its possible involvement in female cancers as well as certain male cancers. Researchers found a single base pair polymorphism (T to C substitution) in the 5'-untranslated region of CYP17. This polymorphism creates a Sp-1 type (CCACC box) promoter site. This base pair also introduces a recognition site for the *Msp*A1 restriction enzyme. The undigested allele is called A1 and the digested allele A2. Much research has been done on these two alleles of CYP17, as they have been implicated in many studies to be involved with the onset of EC, breast cancer, PCOS, prostate cancer and BPH. The A2/A2 genotype is considered 'unfavorable' in breast cancer, while the A1/A1 genotype is over-represented in EC. In males, the A1/A1 genotype indicates an increased risk of developing prostate cancer and BPH.

The relatively low expression of human cytochromes P450 in tissues and organs complicates the study of these proteins. Heterologous expression of these proteins facilitates investigations of enzyme activities and structure/function relationships. The *CYP17* gene has previously been successfully cloned and overexpressed in *P. pastoris* in our laboratory [100]. In this study, we conducted kinetic studies with human CYP17 in *P. pastoris* to determine the kinetic parameters of this enzyme. Although the kinetic properties of human P450c17 have been previously investigated in COS 1 cells [101] and *E. coli* [82], this was the first time, to our knowledge, that the kinetic properties of human P450c17 were investigated in the yeast, *P. pastoris*. Progesterone was used as substrate and steroid metabolites were analysed by HPLC. Results obtained from this study are compared to previous findings and presented in Chapter six.

CHAPTER SIX

An investigation of the enzyme activity of human Cytochrome P450c17 expressed in the yeast *P. pastoris*

6.1 Introduction

The human *CYP17* gene has been successfully expressed in *E. coli* [81,83,98], *S. cerevisiae* [99,102], COS 1 cells [101,103] and HEK-293 cells [104,71] amongst others. This gene and its product has been extensively studied and characterized in various tissue types. The human *CYP17* gene was previously cloned into the pPIC3.5K vector and transformed into *P. pastoris* GS115 cells in our laboratory [100]. In this study human P450c17 was expressed in *P. pastoris* and the catalytic activity of the expressed enzyme investigated *in vivo* using progesterone as a substrate. Progesterone was chosen as substrate as it has been previously shown that human P450c17 has the unique ability to convert progesterone, not only to 17OHprog, but also to 16OHprog [101]. Together with the K_m, the ratio of 17α-hydroxylase to 16α-hydroxylase activity of human P450c17 would serve as an additional parameter to compare the enzyme activity of human P450c17 expressed in COS 1 cells and *P. pastoris*.

6.2 Methods

6.2.1 Activity assay of human P450c17 in *P. pastoris*

P. pastoris strains GS115/pPIC3.5K-CYP17 and GS115/pPIC3.5K (control) were grown as described in section 4.3.2.4. Progesterone metabolism by live yeast cells (5 x 10^7 cells/ml) was investigated after induction with methanol for 48 h in a total volume of 2 ml in a shaking incubator at 30°C. The cell suspension (4 ml per reaction) was harvested by centrifugation at 2000 x g for 5 min at room temperature. The parent vector strain (GS115/pPIC3.5K) was included to serve as a control. The cell pellets were washed with PBS and resuspended in fresh BMMY (1 ml) induction media. The resulting suspension

was pre-incubated for 15 min at 30°C in an Erlenmeyer flask (25 ml), where after the reaction was initiated by adding an equal volume of the appropriate concentration of progesterone and tritium-labeled progesterone (600,000 cpm) in BMMY media. Six different concentrations (0.25, 0.5, 1, 2, 4 and 8 μM) of progesterone were used to determine the kinetic characteristics of human P450c17 expressed in yeast, *P. pastoris*. The control strain, GS115/pPIC3.5K was treated identically, however, only 8 μM progesterone was added to this reaction mixture. The conversion of progesterone to 17OHprog and 16OHprog was monitored by removing aliquots of incubation medium, in duplicate, at the following time intervals: 0, 5, 12, 20, 25, 30, 40, 50 and 60 min. This conversion assay was repeated three times. Steroids in the culture media were extracted with methylene chloride (100:1 solvent to incubation mixture). The water phase was aspirated and the methylene chloride was evaporated under a stream of nitrogen gas. The dried steroids were redissolved in methanol for analysis by HPLC. The HPLC apparatus (Thermo Separation Products, P4000) was equipped with an in-line UV-detector (Thermo Separation Products, UV6000LP) and a radioactive detector (Radiomatic, Tampa, FL). An isocratic separation of the steroids was achieved on a reversed phase C18 column (Phenomenex) with a mobile phase consisting of a methanol and water mixture (75:25) over a period of 35 min at a flow rate of 1 ml/min. All solvents used for the separation of the steroids were filtered through a 0.45 μm pore size filter (Millipore) and degassed by sonication. The substrate, progesterone, as well as the two products, 17OHprog and 16OHprog, were injected as standards to determine the elution time of these steroids in this system. Detection and quantification of the steroids were done by radioactivity. Product formation or substrate utilization was calculated as follows. The amount of radio-activity (cpm) in each compound separated by HPLC, was obtained from the liquid scintillation spectrometer. The total number of counts for all the compounds detected was added and the percentage of each component calculated. Initial reaction rates were determined by a least squares method. Total protein content for each incubation was determined using the bicinchoninic acid method (BCA) (Pierce Chemicals Co.). The K_m as well as the v_0 values were determined by non-linear regression using GraphPad Prism

version 4.00 for Windows[6]. Table 6.1 presents the K_m as well as the v_0 obtained for 16OHprog, 17OHprog and progesterone, respectively.

6.3 Results

6.3.1 Conversion of progesterone to 16OHprog and 17OHprog by P450c17

The elution profile of a separation of steroid standards, progesterone, 17OHprog and 16OHprog is shown in Fig. 6.3.1 (A), while a typical separation of a mixture of steroids obtained after incubation of progesterone with the *P. pastoris* expressing human CYP17 is given in Fig. 6.3.1 (B). The 16OHprog eluted first at approximately 6 min, 17OHprog between 12 and 15 min and progesterone eluted after 20 min (Fig. 6.3.1A and B).

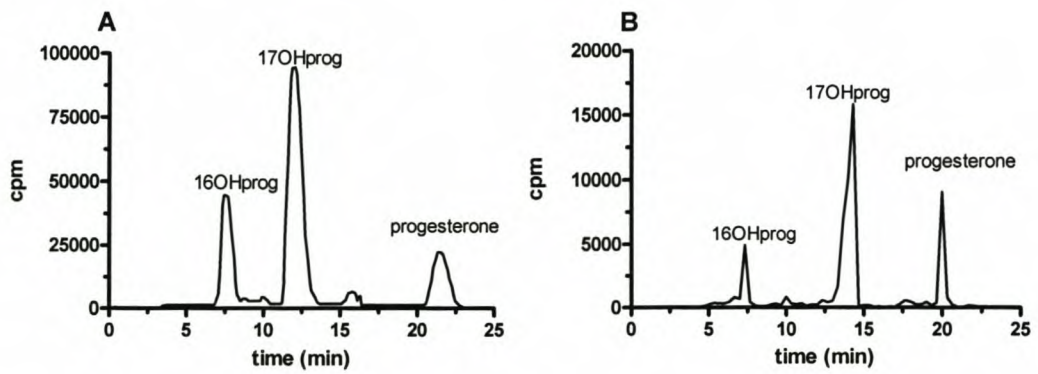

Fig. 6.3.1. HPLC profiles of steroid standards (A) and steroid metabolites (B) formed by the metabolism of radiolabeled progesterone by human P450c17 expressed in *P. pastoris* cells.

P. pastoris cells, transformed with human P450c17, converted progesterone to 16OHprog and 17OHprog (Fig. 6.3.1 (B)). A 60 min time course study of progesterone (8 μM) metabolism is given in Fig. 6.3.2. The ratio of 16OHprog:17OHprog produced was approximately 1:5 across the range of progesterone concentrations (0.25 to 8 μM). When 16OHprog or 17OHprog production was used as a measure of the reaction rate, K_m values of 2.89 and 3.10 μM, respectively, were obtained (Fig. 6.3.3 and 6.3.4, Table 7.1).

[6] GraphPad Software, San Diego California USA, www.graphpad.com.

The overall apparent K_m for progesterone utilization by the human P450c17, expressed in *P. pastoris* cells, was 2.54 µM (Fig. 6.3.5).

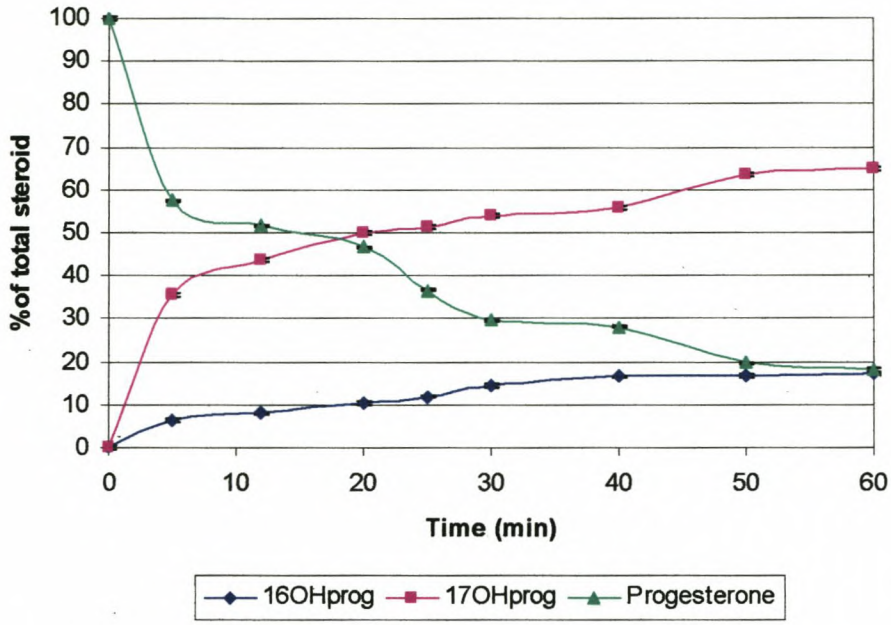

Fig. 6.3.2. Metabolism of progesterone (8 µM) by human P450c17 expressed in *P. pastoris*. Results are representative of three independent experiments.

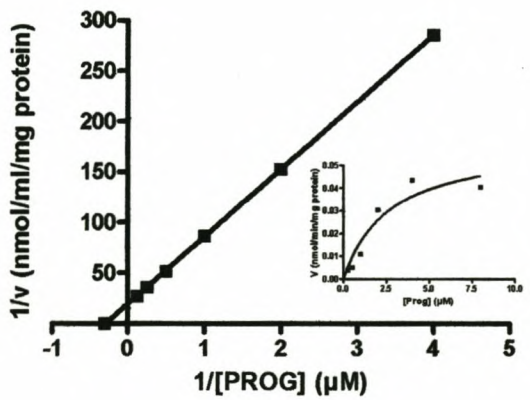

Fig. 6.3.3. Kinetics of 16α-OH-progesterone metabolism by human P450c17 expressed in *P. pastoris* cells (apparent K_m = 2.89 µM; v_0 = 0.27 nmol/min/mg protein). Results are representative of at least three independent experiments.

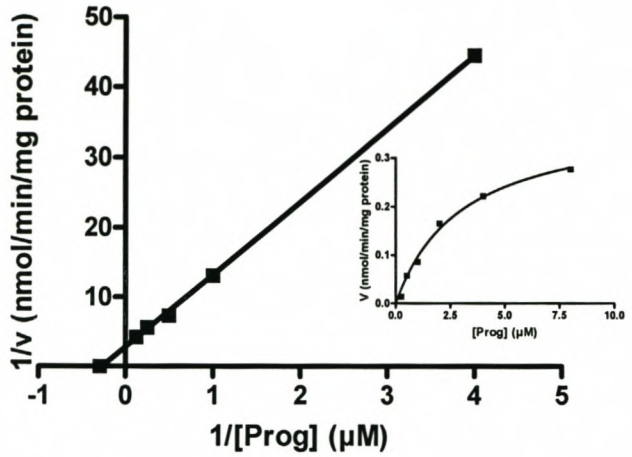

Fig. 6.3.4. Kinetics of 17OHprog metabolism by human P450c17 expressed in *P. pastoris* cells (apparent K_m = 3.10 µM; v_0 = 0.42 nmol/min/mg protein). Results are representative of at least three independent experiments.

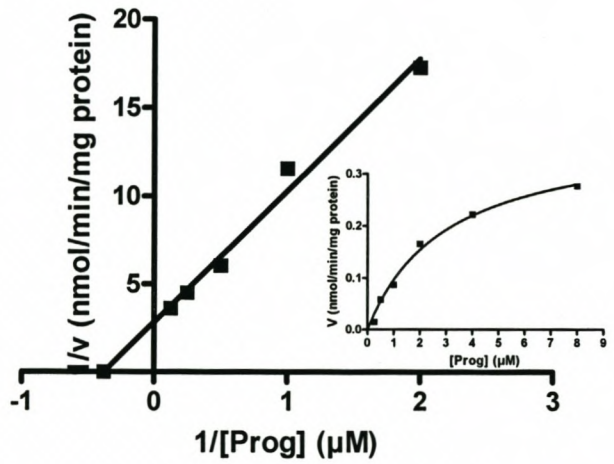

Fig. 6.3.5. Kinetics of progesterone metabolism by human P450c17 expressed in *P. pastoris* cells (apparent K_m = 2.54 µM; v_0 = 0.44 nmol/min/mg protein). Results are representative of at least three independent experiments.

Table 6.1 presents the K_m as well as the v_0 obtained for 16OHprog, 17OHprog and progesterone, respectively.

Table 6.1 Summary of kinetics of progesterone metabolism by human P450c17 expressed in *P. pastoris* cells

Substrate or product	K_m for prog (μM)	v_0 (nmol/min/mg protein)
16OHprog	2.89 ± 0.11	0.27 ± 0.11
17OHprog	3.10 ± 0.05	0.42 ± 0.06
Progesterone	2.54 ± 0.03	0.44 ± 0.02

Results are the average of three experiments ±SD

6.4 Discussion

P450c17 has dual activity, catalyzing both 17α-hydroxylase and 17, 20-lyase reactions. P450c17 is closely associated with the production of estrogens *via* aromatase. As a consequence this enzyme has been studied for its possible involvement in female cancers as well as certain male cancers. It has been implicated in the onset of EC, breast cancer, PCOS and higher levels of serum estradiol in women [63,84,85,91,92,96,97]. In men, it has been associated with increased risk of prostate cancer, BPH and male-pattern baldness [85].

The human *CYP17* gene was previously cloned into the pPIC3.5K vector and transformed into *P. pastoris* GS115 cells in our laboratory. P450c17 was expressed in *P. pastoris* as a functional enzyme [100]. The higher expression levels of P450c17, compared to aromatase, can be attributed to possible multi-integration of CYP17 into the yeast genome[7]. The *P. pastoris* expression system was capable of supporting all of the known activities of the human P450c17 previously shown in COS 1 cells [101]. The expressed P450c17 enzyme converted progesterone to 17OHprog and 16OHprog. Maximum conversion of progesterone (~80 %) to its products was reached after 1 h. The reaction was followed for an additional 2 h, however, 100 % conversion of progesterone

[7] Kolar, N.W. Department of Biochemistry, University of Stellenbosch, Stellenbosch *Personal correspondence*

was not achieved. Incomplete substrate conversion can be attributed to the following: (a) the dissolved oxygen supply was insufficient in the small 25 ml Erlenmeyer flasks used for incubation and could not sustain a high rate of substrate conversion or (b) the available reducing equivalents supporting substrate conversion was insufficient. The 16OHprog product, is characteristic of the human P450c17 enzyme, with 16α-hydroxylation of progesterone by P450c17 being identified only in humans [101]. The ratio of 16OHprog to 17OHprog formed has previously been shown to be 1:4 for the human P450c17 expressed in COS 1 cells [101]. The ratio obtained in the yeast cells was approximately 1:5 (16OHprog:17OHprog). The human P450c17 enzyme expressed in COS 1 cells apparently has a greater affinity for progesterone (~ 0.77 μM) [101] compared to the yeast cells (~ 2.82 μM), while the yeast cells have a greater affinity for progesterone compared to *E. coli* cells (~6.3 μM) [82]. It is possible that K_m could not be assessed accurately in *P. pastoris* due to the fact that oxygen and/or reducing equivalents could have been limiting would influence the accurate assessment of v_0 and hence K_m. The kinetic constants for human P450c17 expressed in COS 1 cells and *P. pastoris* differ markedly under the conditions used in this study. Future experiments will focus on P450c17 expression in *P. pastoris* supplementing reducing equivalents and increasing the oxygen supply to obtain more accurate v_0 values.

Conclusion

Cytochrome P450 enzymes are a large family of heme-containing enzymes. Their characteristic enzymatic function defines them as monooxygenases [1-3]. This group of enzymes plays a crucial role in the detoxification of exogenous xenobiotics and is also responsible for the endogenous metabolism of steroids, vitamins, fatty acids and eicosanoids. Cytochrome P450-dependent enzymes are predominantly found in the ER, however, they are also present in most other tissues. These enzymes can be divided into two main groups, depending on their location, i.e. either microsomal or mitochondrial [2,3]. The steroidogenic cytochrome P450-dependent enzymes are found in the adrenal cortex, testis, ovary, placenta, adipose tissue and brain [2,4,5,7]. The precursor of all steroids is cholesterol and for its conversion a sequence of cytochrome P450-dependent monooxygenation reactions, together with Δ-5 to Δ-4 isomerization coupled to NAD-dependent oxidation of the 3β-hydroxyl function, must take place. The first step in steroidogenesis, conversion of cholesterol to pregnenolone, occurs in the mitochondria and is catalysed by cytochrome $P450_{scc}$. Pregnenolone then moves from the mitochondria to the microsomes, where three microsomal cytochrome P450 enzymes are involved. P450c17 is responsible for the 17α-hydroxylation of pregnenolone or progesterone to produce 17OHpreg or 17OHprog, respectively. The second reaction catalysed by P450c17 is a 17, 20 oxygenase dependent cleavage (lyase reaction) of either 17OHpreg or 17OHprog to yield DHEA or androstenedione. P450c21 catalyses a 21-hydroxylation reaction, with either progesterone or 17OHprog as substrates leading to the formation of deoxycorticosterone or 11-deoxycortisol, respectively. Aromatase is the third microsomal cytochrome P450 involved in steroidogenesis. Three sequential oxygenation reactions are catalysed by this enzyme to convert C19 androgens to C18 estrogens, i.e. androstenedione to estrone or testosterone to estradiol [2,5,6,8,9,14,15,30]. The first two reactions catalysed by aromatase are hydroxylations that take place on position C19 of androgens. The third step, aromatization of the steroid A-ring, is brought about by the oxygen activation. Aromatase is a single enzyme complex and all the mentioned reactions of this enzyme are carried out at the same active site.

Female mortality rates worldwide are significantly influenced by breast, endometrium and ovarian cancers [11,39,40,45-48,53,58]. Many researchers detected aromatase activity in breast cancer, implicating estrogens, the product of aromatase metabolism, in playing a role in the development of breast carcinomas. In hormone-dependent cancers, a continuous supply of estrogen is necessary for the initiation and promotion of the tumor. The mRNA encoding for human aromatase has been localized in peripheral tissues in previous studies [55,70]. To date aromatase protein levels in these tissues have not been determined and there is still much debate over the exact localization of this protein in breast tissue (stroma vs. epithelia) [69,70]. An economic and effective method for the production of aromatase will be valuable in aiding research on the levels, mechanism of action and activity of aromatase in breast cancer. By over expressing the human *CYP19* gene in *P. pastoris*, the expressed aromatase can be used to raise antibodies for immunohistochemical studies that could ultimately lead to the determination and localization of aromatase in normal and cancerous breast tissue *via* ELISA and immunocytochemistry.

Since P450c17 is closely associated with the production of estrogens *via* aromatase, it has been extensively studied for its potential involvement in female as well as certain male cancers. The onset of breast cancer, EC, PCOS and higher levels of serum estradiol levels in women have been linked to P450c17, while in men, it has been implicated in BPH, male-pattern baldness and an increased risk of prostate cancer [63,84,85,91,92,96,97].

Unambiguously, cytochromes P450 play an essential role in biological processes in humans, maintaining health and well being of the individual. Mutations or malfunctioning of CYP genes encoding cytochromes P450, may give rise to human diseases. We are still at the early stages of understanding the many roles of cytochromes P450's in human homeostasis. Therefore, the challenge is to better define their role in metabolism and to develop methods for determining their expression levels and activities *in vivo*. Since aromatase and P450c17 have been linked to these above mentioned disease states, it would be valuable to have a heterologous expression system producing these proteins in sufficient amounts for further studies including kinetic assays, enzymology,

immunohistochemistry and immunocytochemistry. Consequently, sufficient heterologous expression is essential in cytochrome P450 research, since these proteins are only expressed at low levels in tissues making them extremely difficult to study. The heterologous expression of these proteins, allows the determination of enzyme activities in foreign environments, as well as the determination of structure/function relationships associated with cytochromes P450 by site-directed mutagenesis. Furthermore, heterologous expression of human cytochromes P450 will aid in evaluation of the effects of xenobiotics and inhibitors on these enzymes. Sufficient pure cytochrome P450 proteins can be expressed for antibody production, to be used in the localization of aromatase in breast tissue, for example. Nevertheless, heterologous expression can have numerous problems since it is a complex process. Problems are not only encountered at the level of protein stability but also at transcriptional level since the insertion of a foreign gene into an expression vector or multi-copy integration into the genome of the host cell, does not guarantee a high expression level [74,104].

In this study, the human *CYP19* gene, coding for aromatase, was integrated into the yeast *P. pastoris*'s genome. It was possible to induce the transformed *P. pastoris* cells with methanol to express the aromatase enzyme. The assumption can be made that only one copy of the *CYP19* gene was integrated, since transformed cells only exhibited resistance against a G418 level of 0.25 mg/ml, which is indicative of single copy genome uptake. However, possible retention of the protein in the yeasts' ER may also be a reason for the lower expression of aromatase compared to P450c17 in *P. pastoris* [100][8]. Nonetheless, the recombinant *P. pastoris* cells converted androstenedione to estrone indicating the expression of a functionally active enzyme in *P. pastoris.* The endogenous yeast reductase system was therefore capable of electron transport to the expressed human aromatase. The conversion of androstenedione to estrone by aromatase reached equilibrium after 2 h, with only 30 % of the substrate converted to product. P450c17 converted within 80 % of the substrate, progesterone, to 17OHprog and 16OHprog. This incomplete conversion of substrate in both cases can be ascribed to two factors; (a) the

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supply of dissolved oxygen in the small 25 ml Erlenmeyer incubation flasks, was insufficient and/or (b) the reducing equivalents available were inadequate. The assay may not be an accurate reflection of the v_0 value and subsequently the K_m, since P450c17 did not accomplish 100 % conversion. In future studies with these two cytochrome P450 enzymes, aromatase and P450c17, expressed in *P. pastoris*, the oxygen supply should be increased and monitored while reducing equivalents are supplied. This may lead to an increase in product being produced by aromatase and/or allow P450c17 to achieve maximum conversion.

It was possible to isolate and purify aromatase from the membrane fraction by IMAC. With all the methods for expression and purification in place and the newly developed HPLC based aromatase assay, it will be economical and efficient to produce and purify large amounts of protein and conduct kinetic studies with aromatase. Higher expression levels may be achieved by modifying the expression vector, pPIC3.5K-CYP19, to yield a higher copy number. Supplementing reducing equivalents and increasing the oxygen supply during expression may result in more substrate being converted to product. Different approaches such as truncating the amino terminal end of human *CYP19* or another transformation strategy into yeast might also prove useful. Since the structure of aromatase is not well-known and the antigenic determinant has not been determined, the use of synthetic peptides to raise antibodies will be time-wasting and expensive. The production of sufficient amounts of aromatase can be used for future enzymatic studies as well as the production of polyclonal and monoclonal antibodies, allowing the identification and localization of aromatase in breast tissue.

With the *P. pastoris* expression system it was possible to study the activity of the expressed P450c17. The results obtained in this study indicate that *P. pastoris* is a suitable heterologous expression system for the production of active unmodified human aromatase and P450c17. The success of over-expressing and producing unmodified human steroidogenic cytochrome P450 enzymes in *P. pastoris* can assist in studying the cytochrome P450 enzymes to investigate an array of parameters. Potential applications in cytochrome P450 research can be exploited such as large-scale cytochrome P450

production under well controlled conditions as well as enzyme/inhibitor interactions in pharmaceutical applications. The cytochrome P450 content, endogenous b_5 and cytochrome oxidase should be investigated as all these accessory proteins might impact on this heterologously expressed steroidogenic cytochrome P450 to fully understand the cellular environment of *P. pastoris*. The *P. pastoris* expression system is, without doubt a viable expression system for producing unmodified human steroidogenic cytochrome P450 enzymes and may also prove useful in the expression of other non-steroidogenic cytochromes P450.

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