Site-directed mutagenesis of the steroid-15β-hydroxylase (CYP106A2) from *Bacillus megaterium* ATCC 13368 to alter the regio-selectivity of progesterone hydroxylation

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Frau M.Sc Kim Thoa Nguyen

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CONTENTS

ABBREVIATION

Nomenclature of cytochrome P450:

CYP106A2	15β-hydroxylase cytochrome P450 or P450-meg

Abbreaviation:

A	Ampere
AdR	Adrenodoxin reductase
Adx	Adrenodoxin
ATCC	American Type Culture Collection
bp	Base pair
BSA	Bovine serum albumin
C	Celsius
CO	Carbon monoxide
CYP	Cytochrome P450
Da	Dalton
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxy-NTPs
DTE	Dithioerythritol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavine adenine dinucleotide
FMN	Flavine mononucleotide
hr	Hour (s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
K	Potassium
IPTG	Isopropyl-β-D-thiogalactoside
min	Minute (s)
NADPH	Nicotinamide adenine dinucleotide phosphate

nm	Nanometer
PAGE	Polyacrylamid gel electrophoresis
PCR	Polymerase chain reaction
Pfu	*Pyrococcus furiosus*
PMSF	Phenylmethylsulfonyl fluoride
rpm	Round per minute
SDS	Sodium dodecylsulfate
sec	second (s)
TEMED	*N,N,N',N'*-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
UV/vis	Ultraviolet and visible wavelength range
V	Voltage (s)
WT	Wild type

Standard abbreviations for amino acids:

A	Ala	Alanine	L	Leu	Leucine
R	Arg	Arginine	K	Lys	Lysine
N	Asn	Asparagine	M	Met	Methionine
D	Asp	Aspartic acid	F	Phe	Phenylalanine
C	Cys	Cysteine	P	Pro	Proline
Q	Gln	Glutamine	S	Ser	Serine
E	Glu	Glutamic acid	T	Thr	Threonine
G	Gly	Glycine	V	Val	Valine
H	His	Histidine	W	Trp	Tryptophan
I	Ile	Isoleucine	Y	Tyr	Tyrosine

ABSTRACT

CYP106A2 is known as 15β-hydroxylase, but also shows 11α-hydroxylase activity for progesterone, an important pharmaceutical compound. This work focused on the production of a regio-selective hydroxy derivative of progesterone at the C-11 position using site directed mutagenesis of CYP106A2. Based on the docked progesterone-CYP106A2 model, a saturation mutagenesis library (13100 transformants) at positions A395 and G397 had been created to verify the effect of these residues on the regio-selective hydroxylation. Forty of these transformants were randomly selected and screened for higher 11α-hydroxyprogesterone production. Mutants A395I and A395W/G397K showed the highest 11α-hydroxyprogesterone production (19.5 and 56.4-fold, respectively) despite a lower catalytic activity as compared with the WT. Therefore, these mutants were further mutated to improve their catalytic efficiency. Eight new mutants were found to have a higher turnover rate along with an improved 11α-hydroxyprogesterone production. Mutants A106T/A395I and T89N/A395I showed the highest amounts of 11α-hydroxyprogesterone, 2.8- and 4.4-fold increased, respectively, compared with the parent mutants, and 54.9- and 85.2-fold, respectively, compared with the WT. Interestingly, the *in vivo* conversion of progesterone with the mutant T89N/A395I nearly produced the 11α-hydroxyprogesterone. Since the 11α-hydroxyprogesterone exhibits an antiandrogeneic activity and plays a role in the regulation of the blood pressure, the mutant has a potential scope as biocatalyst.

ZUSAMMENFASSUNG

CYP106A2 ist als 15β-Hydroxylase bekannt, zeigt aber auch 11α-Hydroxylase Aktivität für Progesteron. Ziel dieser Arbeit war die Produktion regio-selektiver Hydroxyderivaten des Progesterones an Position C-11 mittels zielgerichteter Mutagenese am CYP106A2. Eine 13100 Transformanten umfassende Mutantenbank, hergestellt mittels Sättigungsmutagenese, ausgehend von einem mit Progesteron gedockten Modell, diente als Grundlage. Zur Untersuchung des Einflusses der Aminosäuren auf die Regio-selektivität wurden 40 Transformanten zufällig ausgewählt und auf eine höhere 11α-Hydroxyhydroxylierung gescreent. Die Mutanten A395I und A395W/G397K zeigten, verglichen mit dem WT, die höchste 11α-Hydroxyprogesterone Produktion (19,5- bzw. 56,4-fach), trotz geringerer katalytischer Aktivität. Diese Mutanten wurden nochmals mutiert, um die katalytische Effizienz zu erhöhen. Acht neue Mutanten mit einer höheren Umsatzrate und verbesserter 11α-Hydroxyprogesterone Produktion wurden gefunden. Die Mutanten A106T/A395I und T89N/A395I bildeten 2,8- bzw. 4,4-mal mehr 11α-Hydroxyprogesteron als die jeweilige Ausgangsmutante und sogar 54,9- bzw. 85,2-mal mehr als der WT. Interessanterweise hydroxylierte die Mutante T89N/A395I während des *in vivo* Umsatzes Progesteron fast ausschließlich an der 11α-Position. Da 11α-Hydroxyprogesteron eine antiandrogene Aktivität zeigt und eine Rolle bei der Regulierung des Blutdrucks spielt, ist zu sagen, diese Mutanten einem großer Erfolg und eine gute Vorraussetzung zur biotechnologischen Anwendung ist.

SUMMARY

Nowadays, steroids are widely used as drugs for the treatment of a variety of diseases. However, the production of the steroids and their derivatives is difficult by chemical methods due to the necessity for regio- and stereo-selective modifications. On the other hand, bacterial enzymes are reported to play an important role in performing biotransformations at specific positions.

CYP106A2 from *Bacillus megaterium* ATCC 13368, one of the few known bacterial steroid hydroxylating enzymes, is able to hydroxylate a variety of 3-oxo-Δ^4-steroids such as testosterone, 11-deoxycorticosterone or 11-deoxycortisol at 15β-position. It converts progesterone mainly into 15β-hydroxyprogesterone and into minor products such as 11α-, 9α-, 6β-hydroxyprogesterone. The aim of this work was to create mutants, which could alter the stereo- and regio-selectivity of progesterone hydroxylation towards the 11α-position.

The starting point was the work described in the Ph.D. Thesis of Lisurek in which 2 residues, A395 and G397, of CYP106A2 were predicted to have an effect on the regio-specificity of the progesterone hydroxylation. These residues were mutated to the corresponding residues contained in CYP11B1 and produced mutants with an increased 11α-hydroxyprogesterone production (Lisurek, 2004). Subsequently, a saturation mutagenesis at positions A395 and G397 was created by Virus, which contained 13100 transformants (Virus, 2006). To identify the role of these 2 residues on the regio-selectivity of progesterone hydroxylation as well as to select the mutant which produced high amounts of the 11α-hydroxy product, 40 transformants from the saturation mutagenesis library were randomly selected. These mutants were expressed in *E.coli* JM109 cells. As predicted, these selected mutants showed a diverse distribution of hydroxylated progesterone products. All mutants increased the minor 11α-, 9α- and 6β-hydroxyprogesterone production as compared with those of the CYP106A2 wild type. Especially, mutants CYP106A2 A395K/G397N and CYP106A2 A395W/G397K showed a higher amount of 11α-hydroxyprogesterone relative to 15β-hydroxyprogesterone, up to 13.5 and 56.4-fold, respectively, compared with that of CYP106A2 wild type. Furthermore, these mutants also exhibited a higher 6β-hydroxyprogesterone formation, approximately 10-fold in comparison with that of the wild type enzyme. Mutant CYP106A2 A395R/G397K converted progesterone mainly in 9α-position, showing a

1.26-fold increase relative to 15β-hydroxyprogesterone. Unfortunately, the catalytic activity of mutant CYP106A2 A395W/G395K, which showed a drastic shift of the regio-selectivity of hydroxylation from C-15 to C-11, was lower than 50% compared with that of CYP106A2 wild type. Similarly, mutant CYP106A2 A395I, obtained by Virus (Virus, 2006), lead to 97.2% of 11α-hydroxylated product but showed 50% less turnover rate than CYP106A2 wild type. Therefore, these 2 mutants, CYP106A2 A395I and CYP106A2 A395W/G397K, were chosen for further mutation by site directed mutagenesis to improve the catalytic efficiency.

The 2 parent mutants CYP106A2 A395I and CYP106A2 A395W/G397W were mutated directly at positions which were reported in the literature to increase the catalytic activity. Thirty new mutants were obtained and coexpressed with pBAR_Twin, containing the cDNAs of AdR and Adx. The fluorescence assay was applied for the screening (Appel *et al.*, 2005; Hannemann *et al*., 2005). Only 13 new mutants showed a higher relative fluorescence than the parental mutants. These 13 mutants were expressed and purified. The 11α-hydroxylation activity of mutants CYP106A2 A106T/A395W/G397K, CYP106A2 A243V/A395I, CYP106A2 T248V/A395I showed improvement of 1.59-, 2.49- and 3.1-fold compared with the 15β-hydroxylation activity of CYP106A2 wild type. These mutants also showed an improvement in the regio-selectivity at 11α-position by a factor of 1.5, 1.5 and 1.2, respectively. Especially, mutants CYP106A2 D217V/A395I, CYP106A2 A106T/A395I/R409L and CYP106A2 F165L/A395I showed an elevation of the 11α-hydroxylation activity (by a factor of 7.1, 7.1 and 9.4, respectively) as compared with the 15β-hydroxylation activity of CYP106A2 wild type. They also increased the 11α-production up to 1.2-, 2.51- and 1.38-fold, respectively, as compared with the single mutant CYP106A2 A395I. Interestingly, 2 mutants CYP106A2 A106T/A395I and CYP106A2 T89N/A395I produced 11α-hydroxyprogesterone in 2.8- and 4.4-fold higher amounts as compared with the parental mutant CYP106A2 A395I, and in 54.9- and 85.2-fold higher amounts as compared with the wild type enzyme. However, these 2 mutants showed only a tiny increase of the catalytic efficiency of 11α-hydroxylation (2.6- and 1.6-fold) as compared with CYP106A2 wild type catalyzed 15β-hydroxylation. Eight mutants, which showed a high catalytic rate along with a prominent 11α-hydroxylation activity, were then selected for *in vivo* progesterone conversion. The distribution of the hydroxylated progesterone products was observed to be different when comparing the *in vitro* and *in vivo* conversion. Except for mutants CYP106A2 A106T/A395I, CYP106A2

F165L/A395I and CYP106A2 A395W/G397K which showed slightly less 11α-hydroxyprogesterone formation, the others showed higher yields of this product during *in vivo* compared with the *in vitro* conversion. The most interesting result was achieved for the progesterone hydroxylation of mutant CYP106A2 T89N/A395I. This mutant converted progesterone mainly at 11α-position. If 11α- hydroxyprogesterone was set to 100%, the ratios of 15β-, 9α- and 6β-hydroxyprogesterone produced by this mutant were 6.0%, 9.7% and 8.0%, respectively. Furthermore, the *in vivo* catalytic activity of mutant CYP106A2 T89N/A395I was faster than the *in vitro* conversion. Thus, it can be stated that a mutant with a completely changed regio-selectivity of hydroxylation (15β → 11α) was obtained being a great success and a prerequisite for a potential biotechnological application.

1. INTRODUCTION

1.1. Steroid hormones

Hormones are defined as organic substances which play a role as chemical messengers produced by organs or tissues such as the endocrine glands, the digestive tract, the heart, and the kidneys, or even the nerve cells. These substances are secreted by certain cells, liberated directly into the bloodstream and transported to a distant part or parts of the body where they exert a specific effect in extremely slight amounts. In the human body, they are responsible for regulation of metabolism, stimulation or inhibition of growth, activation or inhibition of the immune system, induction or suppression of apoptosis, preparation of the body for activities. Most hormones are subdivided chemically in 3 main classes: peptides, amines and steroids. However, some messenger substances such as the eicosanoids act similarly to hormones but act locally and can belong to a fourth group (Lehninger *et al*., 1998).

The peptide hormones consist of 3 to more than 200 amino acids, involve all hormones of the hypothalamus, the hypophyse as well as the pancreas (e.g. insulin and glucagon). The amine hormones are the derivatives of amino acids, e.g. tyrosine (adrenaline, noradrenaline and thyroxin) which are produced e.g. in the medulla of the adrenal glands. The class of the fat soluble steroid hormones is primarily synthesized in the adrenal cortex and the gonads. They are generally carried in the blood bound to specific carrier proteins, for instance, sex hormone-binding globulin (Lehninger *et al*., 1998). Steroid hormones are composed of three six-membered rings and one five-membered ring, called perhydrocyclopentanophenanthren, which defines the “steroid nucleus” (Figure 1.1). These fat soluble steroid hormones are derived from cholesterol. They are able to pass through the cell membrane and can combine easily with a protein receptor. They are subdivided into glucocorticoids, mineralocorticoids, progestins, androgens and estrogens.

Gluco- and mineralocorticoids are synthesized in the adrenal cortex with the participation of cytochromes P450 (Bureik *et al*., 2002). Glucocorticoids and adrenal androgens are synthesized in the *Zona fasciculata/reticularis* whereas mineralocorticoids are synthesized in the *Zona glomerulosa* of the adrenal gland (Miller and Tyrell, 1995). On the other hand, the sexual hormones are made by the testes or the ovaries (Brook, 1995).

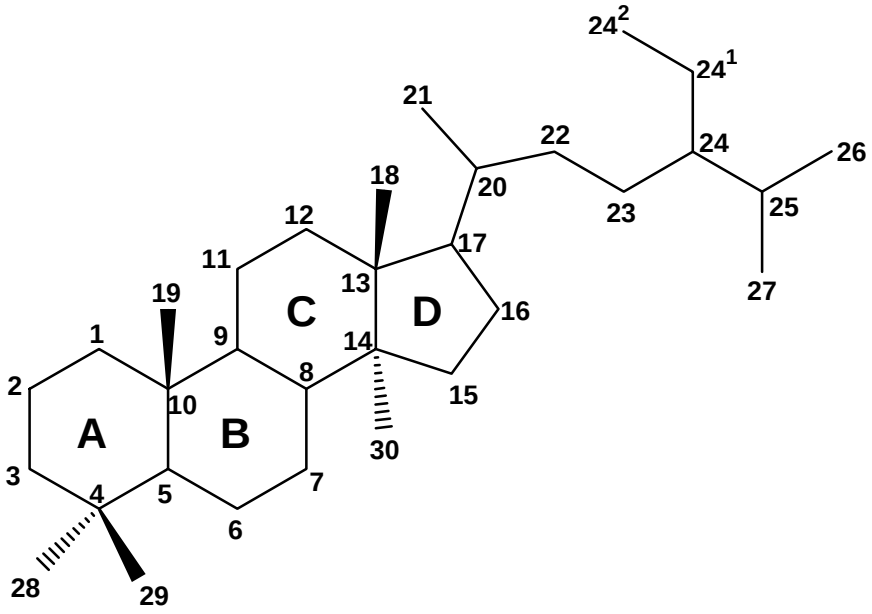


Figure 1.1: The steroid structure containing the "steroid nucleus", which is called perhydrocyclopentanophenanthren, composes of three six-membered rings and one five-membered ring.

The name of the so-called glucocorticoids descends from their role in the regulation of the glucose metabolism, their synthesis in the adrenal cortex and their steroidal structure. Glucocorticoids bind to the glucocorticoid receptor and cause their broad effects via the nuclear receptor affected pathways. The most important glucocorticoids are cortisol and corticosterone, whereby cortisol plays this role in human. Cortisol regulates or supports functions such as cardiovascular, metabolic, immunologic (suppressing the immune answer as well as inflammation routes and allergic reactions) and homeostatic processes. Furthermore, in human, corticosterone plays an important role as an intermediate of the steroidogenic pathway from pregnenolone to aldosterone. Glucocorticoids influence the activity of almost every cell in the body. Glucocorticoids are essential for life but also implicated in the pathogenesis of disease. They cause several adverse effects even though they still have a major place in the treatment of diseases since past 50 years as inflammatory drugs. They also play an important role in the regulation of growth and development of human but their levels are normally maintained within very narrow levels during development. They are required for the normal maturation of the lung and the central nervous system and play a critical role in the control of postnatal growth. Disturbances in the glucocorticoid milieu at critical stages of development may thus have long-term and potentially harmful effects on physiology, thereby influencing disease susceptibility (Buckingham *et al*., 1997; Buckingham, 2006).

Mineralocorticoids are mainly produced in the *Zona glomerulosa* and play a key role in the salt and water metabolisms and in regulating the blood pressure (Fardella and Miller, 1996). The mineralocorticoid receptor is a nuclear receptor that is critical for controlling Na^+ and K^+ transport in epithelial cells, most notably in the kidney and colon (Pearce *et al*., 2003). It is also present in other non-epithelial tissues such as cardiac myocytes, blood vessels, adipose tissue and plays an important role in cardiovascular function, neuronal fate and adipocyte differentiation. It can be activated by both, mineralocorticoids and glucocorticoids (Yang and Young, 2009). The most important mineralocorticoid is aldosterone. Aldosterone is a key regulator of electrolyte and water homeostasis and plays a central role in blood pressure regulation (Escher, 2009). In the absence of mineralocorticoids, the kidney discards Na^+ and retains K^+ and H^+, leading to hyponatremia, hyperkalemia, acidosis, dehydration and death (Miller, 2005). In contrast, increased circulating aldosterone is linked with insulin resistance and impaired glucose homeostasis which can result in the development of endothelial dysfunction, atherosclerosis, and kidney related disease and other disorders. Aldosterone-induced oxidative stress and inflammation cause adverse role in impairing insulin signaling. Consequently, the mineralocorticoid receptor blockade restores insulin sensitivity, counterbalances the deleterious cardiovascular and renal effects of aldosterone, and emerges as an alternative to improve blockade of the renin-angiotensin-aldosterone system. This effect potentially could contribute to reduce the burden of cardiovascular disease (CVD) and chronic kidney disease (CKD) (Lastra-Gonzalez *et al*., 2008). On the other hand, alterations in steroid hormone biosynthesis have been related with a number of pathologies, in particular hormone responsive cancers (such as breast and prostate cancer) and over production of aldosterone is related to cardiac fibrosis and heart failure (Costello-Boerrigter *et al.*, 2007; Funder, 2007; Young *et al.*, 2007; Shapiro *et al.*, 2008).

Sex hormones are essential for sexual differentiation and reproduction with distinct roles in women and men. Reproduction in both sexes is regulated by a hormonal system involving the hypothalamus, the pituitary gland, and the gonads (the testes or ovaries). Sex hormones also have a protective effect on a number of non-reproductive systems such as the immune, cardiovascular and the nervous system and on the bone structure (Evans, 1988; Hess *et al*., 1997; Roselli, 2007; Prins and Putz, 2008). There are 3 classes of sex steroids: progestins, estrogens and androgens. Progestins play a role in the normal menstrual cycle and in the maintenance of pregnancy (Miller, 2005). Androgens regulate

male sex characteristics but serve in both sexes for muscle enhancement, and also for bone strength in case of estrogen-deficiency (Copland *et al.*, 2009). Estrogens, which are responsible for development of female sex characteristics (Ottolenghi *et al.*, 2007), also augment bone strength in both sexes (Schoenau, 2006). Sex steroids mostly circulate bound by albumin and sex hormone-binding globulin (Hammond and Bocchinfuso, 1996).

Several other tissues such as the gastrointestinal tract, the breast, and the immune system, which are not traditionally considered as steroid targets, are also affected by sex steroids. (Enmark and Gustafsson, 1999; Nagai and Brentani, 2008; Guerriero, 2009). For example, estrogen exerts anti-inflammatory effects while progesterone, on the other hand, has been shown to exert pro-inflammatory effects on neutrophils (Bouman *et al.*, 2005).

1.2. Cytochromes P450

1.2.1. General aspects

Cytochromes P450 (abbreviated CYP, P450) are hemoproteins which were discovered over 55 years ago and are broadly distributed in all domains of life. The name cytochrome P450 is derived from the fact that the spectral properties of the *b*-type heme display a typical absorption maximum of the reduced CO-bound complex at 450 nm. Therefore cytochrome P450 is a hemoprotein where P stands for pigments and 450 demonstrates the absorption peak of the ferrous CO complex at 450 nm. This unusual spectral feature is induced by a cystein thiolate group forming the fifth ligand of the heme iron and assigning the P450 enzymes as heme thiolate proteins. P450 enzymes are external monooxygenases, assuming that they need an external electron donor, which transfers the electrons necessary for oxygen activation and the following substrate hydroxylation (Bernhardt, 2006). In general, P450 systems catalyze the following reaction:

$$\mathbf{RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+}$$

Many members of the P450 superfamily of hemoproteins are currently known, and the number grows continuously as more genomes are identified. There are 11614 sequenced P450 genes until November 2009, which are annotated by Nelson (http://drnelson.utmem.edu/cytochrome.html). The amount of P450 genes varies

enormously in different species. For instance, 3282 P450 genes are found in animals, 1015 in bacteria, 22 in archaea and only 2 are discovered in viruses.

There are more than 20 different reactions, which can be catalyzed by P450 enzymes such as hydrocarbon-, aromatic-, N-hydroxylation, N-, O- and S-dealkylation, alkene-, arene-epoxidation, N-, S-oxidation, alkyne oxygenation, oxidative deamination, oxidative and reductive dehalogenations, alcohol and aldehyde oxidations, dehydrogenation, dehydratations, N-oxide reduction, epoxide reduction, reductive β-scission of alkyl peroxides, NO reduction, isomerization and oxidative C-C bond cleavage (Sono *et al*., 1996; Mansuy, 1998; Guengerich, 2001a, b). To coordinate these functions their substrates are also diverse including fatty acid, steroids, prostaglandins as well as a multitude of foreign compounds such as drugs, anaesthetics, organic solvents, ethanol, alkylaryl hydrocarbon products, pesticides and carcinogens (Bernhardt, 2006).

A catalytic cycle of the P450 reaction was proposed in 1968 (Gunsalus *et al.,* 1975; Griffin, 1979) to prove the roles of iron, protein and oxygen, and was updated in 2005 by Denisov *et al.* shown in the following Figure 1.2. According to this, the catalytic turnover of P450 enzyme starts with the binding of substrate to the resting state of the low-spin (LS) ferric enzyme (1), results in altering the spin state to the high spin (HS) substrate bound complex (2) and perturbs the water coordinated as the sixth ligand of the heme iron. The HS Fe^{3+} is reduced to a ferrous state easily (3). In the next step, oxygen binds leading to an oxy-complex (4) which is the final stable intermediate in this cycle. The reduction of this complex by insertion of the second electron produces the peroxo-ferric intermediate (5a) and its protonated form the hydroperoxo-ferric intermediate (5b). Subsequently, ferric hydroperoxy is protonated once at the distal oxygen atom and undergoes heterolytic cleavage of the O-O bond to form Compound I (6) and water. This species then attacks the substrate to yield the hydroxylated product and dissociates to let the cycle start again. Additionally, there are three major abortive reactions: (i) autoxidation of the oxy-ferrous enzyme (4) with concomitant production of a superoxide anion and return of the enzyme to its resting state (2), (ii) a peroxide shunt, where the coordinated peroxide or hydroperoxide anion (5a,b) dissociates from the iron forming hydrogen peroxide, thus completing the unproductive (in terms of substrate turnover) two electron reduction of oxygen and (iii) an oxidase uncoupling with oxidation of the ferryl-oxo intermediate to water by a four-electron reduction of the dioxygen molecule (Denisov *et al.,* 2005).

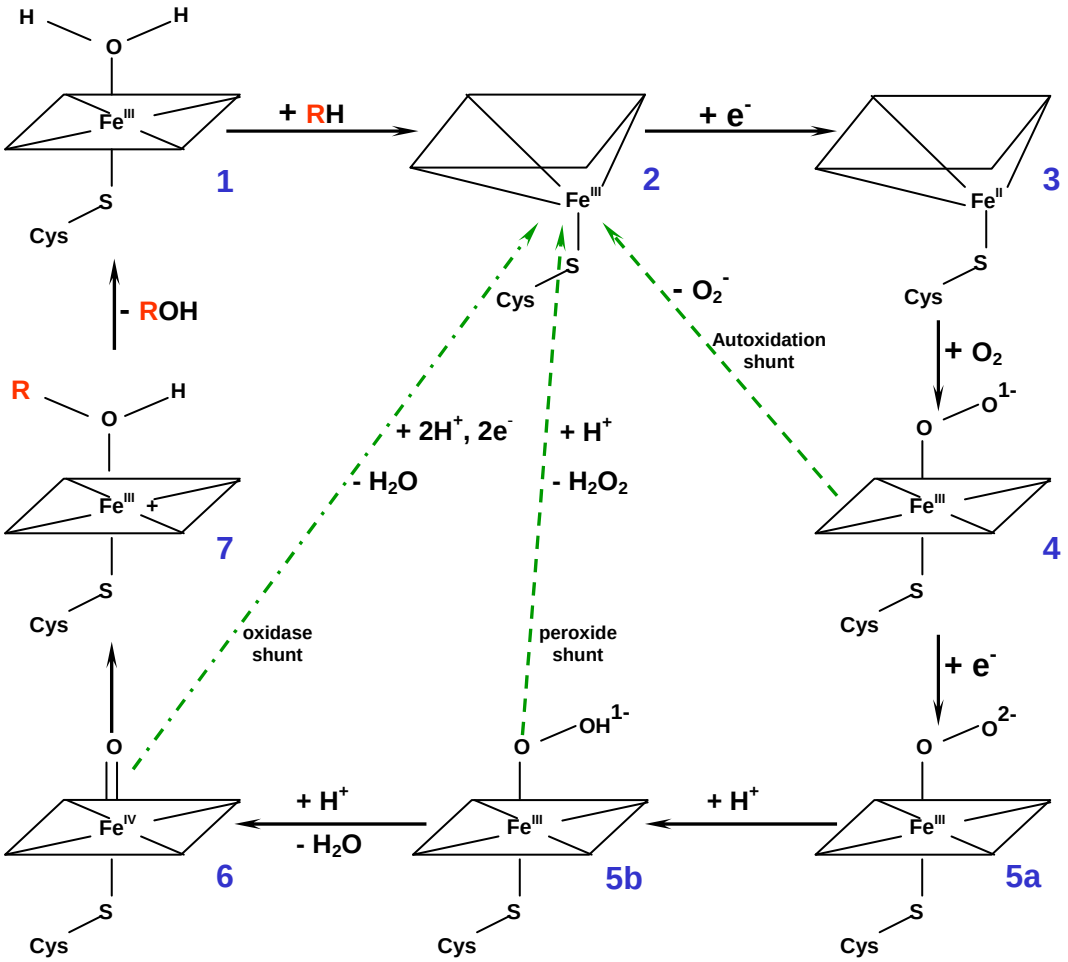


Figure 1.2: Catalytic cycle of cytochrome P450.

1.2.2. Electron transport systems for P450 enzymes

As P450 enzymes are external monooxygenases, they need an external electron donor in the electron transfer chain for substrate conversion (Bernhardt, 2006). In general, Chapman has divided the P450 enzymes into 4 main classes based on the redox partners (http://www.chem.ed.ac.uk/chapman/p450.html). However, in 2007, Hannemann *et al.* updated and classified the electron transport systems for P450 enzymes into 10 classes (Hannemann *et al.*, 2007).

Class I systems involve most bacterial P450 systems as well as the mitochondrial P450 systems from eukaryotes. They are composed of 3 separate proteins: a FAD-containing reductase which transfers reduction equivalents from pyridine nucleotide (NADH or NADPH) to the second component of the system, a ferredoxin, which in turn reduces the P450 enzyme itself (Hannemann *et al.*, 2007). All 3 proteins are soluble in bacteria

(Figure 1.3A). However, only the ferredoxin is a soluble protein of the mitochondrial matrix, whereas the reductase and the P450 are membrane associated and membrane bound to the inner mitochondrial membrane, respectively (Figure 1.3B) (Bernhardt, 1996 and 2006; Gunsalus and Sligar, 1978; Lambeth, 1991; Hannemann *et al.*, 2007).

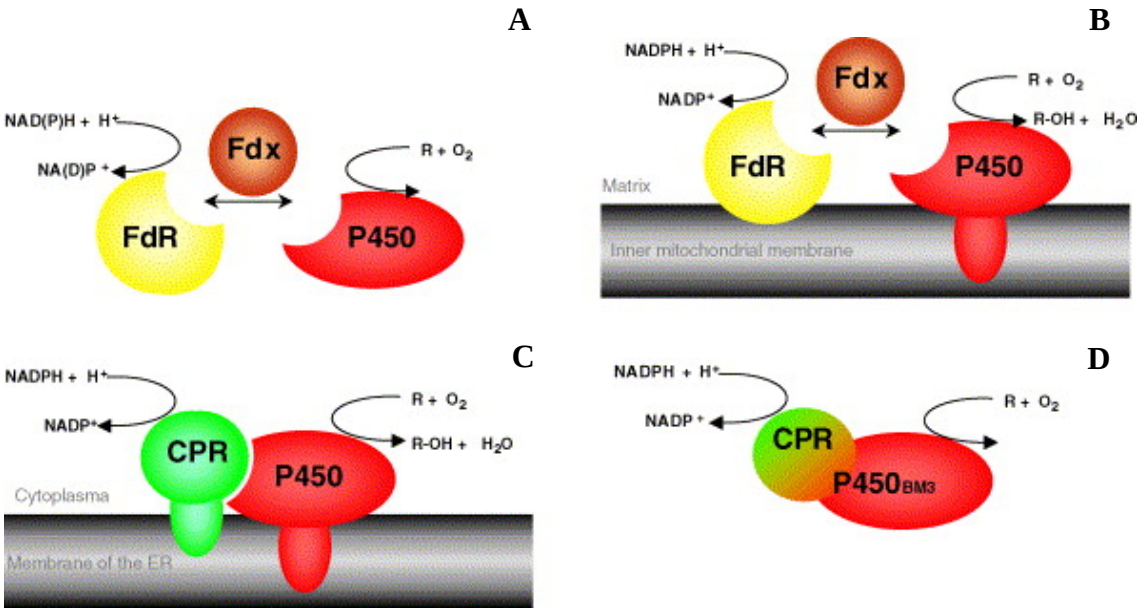


Figure 1.3: Schematic organization of different P450 systems. **A)** bacterial system (Class Ia); **B)** mitochondrial system (Class Ib); **C)** microsomal system (Class II); **D)** self-sufficient CYP102A1 (Class VIII) (Bernhardt, 2006; Hannemann *et al*., 2007).

Class II P450 proteins are popular in eukaryotes and perform extremely diverse catalytic reactions. The monooxygenase system of this class is found in the endoplasmic reticulum (ER) of eukaryotes containing two integral membrane proteins: the P450 enzyme and the NADPH-dependent P450 reductase (Figure 1.3C). The reductase contains the prosthetic groups FAD and FMN, which transfer both required redox equivalents from NADPH to one of the many P450 isozymes (Hanukoglu, 1996).

P450BM-3 (CYP102A1) isolated from *Bacillus megaterium* is a representative of a class VIII P450 (Hannemann *et al.*, 2007). This system consists of P450 proteins, which are fused to their eukaryotic-like diflavin reductase partner – P450 reductase – in a single polypeptide chain and therefore, are catalytically self-sufficient as monooxygenase from NADPH to the component (Figure 1.3D) (Narhi and Fulco, 1986).

1.2.3. Cytochrome P450 structure

The published sequences of P450 proteins reveals that the sequence identity between different P450 proteins (from mammalian to fungal or bacteria) is less than 20% (Hasemann *et al.,* 1995). However, P450 enzymes share a common overall fold and topology. It has been shown that the conserved P450 structural core is formed by a four helix bundle composed of 3 parallel helices termed D, L and I and 1 antiparallel helix E as well as helices J and K (Presnell and Cohen, 1989). In this conserved form, the heme group is confined between the distal I-helix and proximal L-helix and bound to the adjacent cysteine heme ligand loop containing the P450 signature amino acid sequence FxxFx(H/R)xCxG. The absolutely conserved cysteine is the proximal or "fifth" ligand to the heme iron. This sulfur ligand is a thiolate (Dawson *et al.,* 1976) and is the origin of the characteristic name given due to the 450nm Soret absorbance observed for the ferrous CO complex. Typically, the proximal cysteine forms two hydrogen bonds with neighboring backbone amides (Denisov *et al.,* 2005).

The long I-helix forms a wall of the heme pocket and contains the motif (A/G)-Gx(E/D)T which is placed in the middle of the helix. The I-helix also contains a highly conserved threonine that is positioned in the active site and is thought to be involved in catalysis (Imai *et al.*, 1989; Martinis *et al.*, 1989; Kimata *et al.*, 1995; Vidakovic *et al.*, 1998, Kim *et al.*, 2008). The K-helix on the heme proximal side contains the conserved motif Glu-X-X-Arg, which seems to be responsible for the stabilization of the core structure. The substrate binding pocket lies on the A and D pyrrol ring of the heme. There is an enough structural diversity to allow for the binding of substrates of significantly different sizes and with varying degrees of specificity to different P450 enzymes, although the P450-fold is highly conserved (Pylypenko *et al.*, 2003; Zerbe *et al.*, 2002). Gotoh proposed 6 regions that are involved in substrate recognition site (SRS): the B'-helix region (SRS 1), part of the F and G helices (SRS 2 and SRS 3), a part of I-helix (SRS 4), the K-helix β2 connecting region (SRS 6), and the β4 hairpin (SRS 5) line the P450 active site (Figure 1.4). The predetermination of SRSs is helpful for the prediction of point mutations within the SRSs which significantly influence the substrate specificity (Gotoh, 1992). The SRSs are considered to be flexible protein regions, moving upon substrate binding in an induced-fit mechanism to favor substrate binding and subsequently the catalytic reaction (Pylypenko and Schlichting, 2004; Denisov *et al.*, 2005).

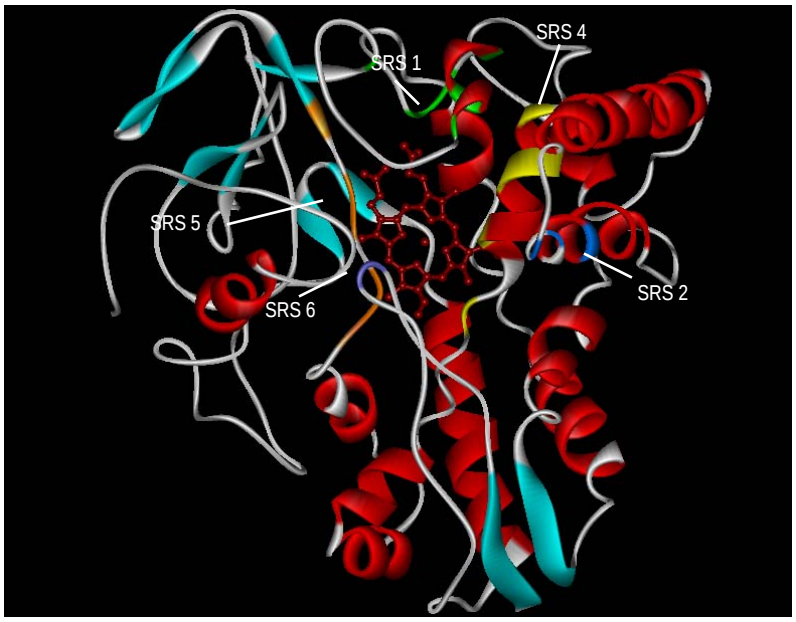


Figure 1.4: The substrate recognition sequence (SRS) regions of CYP106A2: These regions are highlighted in green (SRS 1), in blue (SRS 2), in yellow (SRS 4), in orange (SRS 5) and in violet (SRS 6). SRS 5 is not existed in this enzyme. Heme group is presented in dark red. The CYP106A2 structure is derived from the computer modeling of Lisurek (Lisurek, 2004).

1.2.4. Substrate binding

The interaction between substrate and P450 enzymes has been observed according to the changes of the UV spectra. These changes indicate certain binding characteristics of ligand-P450 complexation reactions. It has been found that there are 3 categories ligand binding: type I, type II and type III (or reverse type I). The difference of ligand binding is used to classify the variety of P450 active site interactions as well as to calculate the modulation of hemoprotein spin state equilibria which can accompany substrate binding (Schenkman *et al.*, 1981; Schenkman and Kupfer, 1982). A type I spectral change entails a reduction in the Soret absorption at 420 nm and a concomitant increase in the 390 nm absorption. In contrast to type I, substrates which exhibit a type II spectral change appear a decrease in absorption at around 390 - 405 nm and an increase at 425 - 435 nm, with an isobestic point at about 419 nm. The third spectral change that of reverse type I, shows an increased absorption of 420 nm and a decrease at 390 nm (Lewis, 1996). In general, it has been seen that the substrates for P450 enzymes are hydrophobic and poorly soluble in water. Substrate binding to P450 is a complex process since this biomolecular recognition event can trigger the change of the spin state from low spin to high spin in the heme iron and concomitantly induces a change in the reduction potential from ca. -300 to a ca. 100

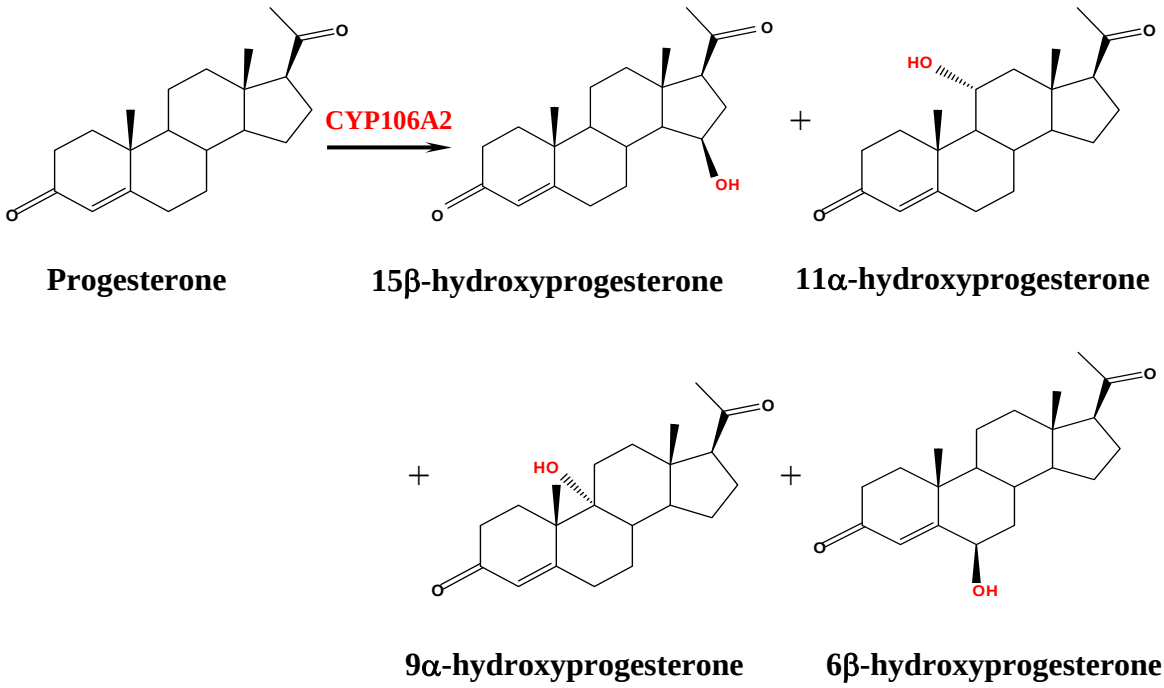
mV more positive value (Guengerich *et al.*, 1975; Williams-Smith and Cammack, 1977; Guengerich, 1983; Daff *et al.*, 1997; Fantuzzi *et al.*, 2004, Denisov *et al*., 2005). Many P450 enzymes have a broad spectrum of possible substrates (for each individual P450). When analyzing of the large structural rearrangement by X-ray crystal structure suggests that the aforementioned substrate recognition sites are flexible and provide substrate access to the heme, which is otherwise buried in the protein globule. In the absence of charged and hydrogen-bonding groups on substrate molecules as well as in the active sites of the most P450 enzymes, such binding mechanisms provide an alternative means for substrate stabilization at the active center (Denisov *et al*., 2005).

1.3. CYP106A2

CYP106A2 from *Bacillus megaterium* ATCC 13368 was first described by Mc-Aleer *et al.* in 1958 (Mc-Aleer *et al.,* 1958). Until 1970s, however, this enzyme was studied continuously in more details by Berg *et al*. They demonstrated the presence of a cytochrome - dependent steroid hydroxylase system in *Bacillus megaterium* (Berg *et al.,* 1975, 1976) and subsequently isolated, characterized, and purified the 15β-hydroxylase to apparent homogeneity (Berg *et al.,* 1979a, b).

The CYP106A2 system is composed of 3 soluble protein components: (i) a reductase (megaredoxin reductase), (ii) a ferredoxin (megaredoxin), and (iii) the cytochrome P450 (CYP106A2, P450-meg). The ferredoxin reductase is strictly NADPH-dependent, it contains the prosthetic group FMN, and has a molecular weight of 55 - 60 kDa. The megaredoxin, which was purified to apparent homogeneity, has a molecular weight of ca. 14 kDa, (Berg *et al.,* 1982). However, it is still unclear whether megaredoxin possesses a [2Fe-2S] cluster or a [4Fe-4S] cluster. The natural function of CYP106A2 is not yet clear. The activity of CYP106A2 can also be reconstituted with bovine adrenodoxin reductase and bovine adrenodoxin (Berg *et al.,* 1979a; Simgen *et al.,* 2000), with putidareductase and putidaredoxin from *Pseudomonas putida* (Agematu *et al.,* 2006), or with *Anabaena* PCC 7119 ferredoxin reductase (FNR) in combination with either *Anabaena* PCC 7119 ferredoxin (Fd) or *Anabaena* PCC 7119 flavodoxin (Fld) (Lisurek, 2004). Moreover, active CYP106A2 was already successfully expressed in *Bacillus subtilis* 168. Therefore, it is assumed that *Bacillus subtilis* 168 possesses an electron transfer system which can support the CYP106A2 activity (Rauschenbach *et al.*, 1993).

The CYP106A2 gene was cloned and sequenced in 1993. It is a soluble protein that consists of 410 amino acids and has a molecular weight of 47.5 kDa (Rauschenbach *et al.*, 1993). It can hydroxylate a variety of 3-oxo-Δ^4-steroids mainly in 15β-position. For example, deoxycorticosterone (DOC), testosterone, and androstenedione are converted into their 15β-hydroxy homologues. However, ring A-reduced, aromatic, and 3β-hydroxy-Δ^5-steroids do not serve as substrates for the 15β-hydroxylase system (Table 1.1) (Berg *et al.*, 1976). Because of the capacity to hydroxylate a large variety of substrates, CYP106A2 becomes an interesting candidate for industrial production of steroids or their intermediates as useful pharmaceutical targets. CYP106A2 converts progesterone not only into 15β- and 6β-hydroxyprogesterone (Berg *et al.*, 1979b) but also into di/poly-hydroxyprogesterone and 2 other products: mono-hydroxyprogesterone at the 11α- and 9α-positions (Figure 1.5) (Lisurek *et al.*, 2004; Kang *et al.*, 2004). In case of the 11-deoxycorticosterone conversion, the substrate binding status was not detectable by UV-Vis spectroscopy. However, an effect of substrate binding has been detected using the CO stretch mode infrared spectrum indicating that 11-deoxycoricosterone binds in the heme pocket near the iron ligand (Simgen *et al.*, 2000).



Progesterone **15β-hydroxyprogesterone** **11α-hydroxyprogesterone**

9α-hydroxyprogesterone **6β-hydroxyprogesterone**

Figure 1.5: Progesterone was converted by CYP106A2 into 4 mono- (15β-, 11α-, 9α-, 6β-hydroxyprogesterone) products.

Table 1.1: Substrates of CYP106A2 from *Bacillus megaterium* ATCC 13368 (*part 1*).

Substance	Conversion	Reference
progesterone	+	Berg et al., 1976, 1979b; Kang et al., 2004 ; Lisurek *et al*., 2004
DOC	+	Berg *et al*., 1976, 1979b
6-fluor-16-methyl-DOC	+	Rauschenbach, 1993
17α-hydroxyprogesterone	+	Berg *et al*., 1976, 1979b
20α-dihydroprogesterone	+	Berg *et al*., 1976, 1979b
androstendione	+	Berg *et al*., 1976, 1979b
testosterone	+	Berg *et al*., 1976, 1979b; Agematu *et al*., 2006
corticosterone	+	Berg *et al*., 1976, 1979b
aniline	+	Berg and Rafter, 1981
estrone	-	Berg *et al*., 1976
estradiol	-	Berg *et al*., 1976
estriol	-	Berg *et al*., 1976
dehydroepiandrosterone	-	Berg *et al*., 1976
pregnenolone	-	Berg *et al*., 1976
5α-androstan-3α,17β-diol	-	Berg *et al*., 1976
5α-dihydrotestosterone	-	Berg *et al*., 1976
5α-androstan-3α,17β-diol	-	Berg *et al*., 1976, 1979b
5α-androstan-3α,17β-diol-3,17-disulphate	-	Berg *et al*., 1976
5β-pregnan-3α,20α-diol	-	Berg *et al*., 1976
cholesterine	-	Berg and Rafter, 1981
desoxycholesterine	-	Berg and Rafter, 1981
cholic acid	-	Berg and Rafter, 1981
desoxycholic acid	-	Berg and Rafter, 1981
β-sitosterol	-	Berg and Rafter, 1981
lithocholic acid	-	Berg and Rafter, 1981
hexadecanoic acid	-	Berg and Rafter, 1981
octadecanoic acid	-	Berg and Rafter, 1981
prostaglandin $F_2\alpha$	-	Berg and Rafter, 1981
biphenyl	-	Berg and Rafter, 1981

Table 1.1: Substrates of CYP106A2 from *Bacillus megaterium* ATCC 13368 (*part 2*).

Substance	Conversion	Reference
benzo[a]pyrene	-	Berg and Rafter, 1981
lidocaine	-	Berg and Rafter, 1981
ethylmorphine	-	Berg and Rafter, 1981
aminopyrine	-	Berg and Rafter, 1981
7-ethoxyresorufin	-	Berg and Rafter, 1981
imipramine	+	Berg and Rafter, 1981
betulin acid	+	Chatterjee *et al*., 2000
betulon acid	+	Chatterjee *et al*., 2000
4-pregnen-20β-ol-3-on	+	Bleif, 2007
17α-methyltestosterone	+	Bleif, 2007
ethisterone	+	Bleif, 2007
4-pregnen-17α,20α,21-triol-3-on	+	Bleif, 2007
melengestrol acetate	+	Bleif, 2007
dihydrochinopimaric acid	+	Bleif, 2007
11-keto-β-boswellic acid	+	Bleif, 2007

The amino acid sequence alignment result indicates that CYP106A2 resembles CYP106A1 (BM-1) from *Bacillus megaterium* 14581 with 63% identity and 76.5% similarity (Rauschenbach *et al.*, 1993). Unfortunately, all attempts to crystallize CYP106A2 have been unsuccessfully yet, probably due to its insufficient stability. However, a model of CYP106A2 was constructed in 2004 by Lisurek *et al.* to gain deeper insight into the structure and other properties such as the catalysis, the stability or the redox-partner binding of CYP106A2. According to the model, in the heme binding site of CYP106A2, the A-ring propionic acid interacts in a bidentate fashion with residue 296, the D-ring propionic acid interacts with residues at positions 96, 353 and 100. Amino acid 247, a strong conserved threonine in the I-helix, is also present in the active site adjacent to the heme and is suggested to be involved in the proton shuttle that is important for proton delivery and molecular oxygen activation (Lisurek, 2004; Lisurek *et al*., 2008).

1.4. Biocatalysis and evolution of the cytochrome P450 superfamily

Cytochromes P450 are ubiquitously distributed enzymes, which possess high complexity and display a broad field of being catalyzed a variety of interesting reactions (Bernhardt, 2006). Therefore, cytochromes P450 become promising candidates as biocatalyst for biotechnological application in the future. Biocatalysis in this era is regarded as a standard technology for the production of chemicals and their derivatives. An analysis of 134 industrial biotransformations reveals that hydroxylases (44%) and redox biocatalysts (30%) are the most prominent categories (Straathof *et al.*, 2002). The use of biological catalysis has been dramatically expanded because of the recent developments in the artificial evolution of genes that code for enzymes (Powell *et al.*, 2001).

Steroids comprise a large class of important chemical and biological compounds. Since 1950, however, steroids have been the traditional field for industrially used microbial transformation using P450 enzymes due to their capability of accepting a wide array of complex molecules as substrates, and their exclusively selective, reactions with unparalleled chiral (enantio-) and positional (regio-) selectivities (Mahato and Garai, 1997; Powell *et al.*, 2001). These attributes have been implemented in various applications, especially in the food and pharmaceutical industries where high reaction selectivity on complex substrates is required (Schmid, 2001). In this context, biocatalysis has the potential to deliver “greener” chemical syntheses (Woodley, 2008). Nowadays, further developmental activities emphasize the application of genetic engineering of microorganisms and directed evolution of enzymes for improvement as steroid-transforming agents (Mahato and Garai, 1997). The engineering of P450 proteins has been performed with several purposes. The first and the most fundamental task have been to enable successfully the recombinant expression in host systems such as bacteria. This in turn led to an effort to solubilize the proteins as a prerequisite for crystallization and structural study (Gilliam, 2008).

1.4.1. Directed evolution

Directed evolution is a useful tool to improve existing properties of enzymes or to develop new features. It does not need any protein structure or any information. An approach to improve a single protein or its parts includes cassette library mutagenesis (involving random, biased, saturation, and codon-based cassette mutagenesis), and random point mutagenesis (including error-prone polymerase chain reaction (PCR),

mutator strains, and UV or chemical mutagenesis). This can introduce (partially) random mutations either into a small selected region of a gene or through a gene (Powell *et al.*, 2001).

The study of directed evolution is always better when wild type enzymes often show an undesirable substrate specificity, or a slight stability (during changing in pH, a temperature or using a different solvent), or a slight or an insufficient activity (Powell *et al.*, 2001). Error prone PCR and gene shuffling methods are frequently applied during directed evolution. Generally, these methods become a powerful tool for improving the utility of the enzymes for industrial applications to have efficient to broaden the enzymatic activity and the substrate specificity. Arnold and coworkers, for example, succeeded in increasing the activity of P450 BM-3 towards the saturated hydrocarbon octane (Farinas *et al.*, 2001) and even the gaseous alkane propane (Glieder *et al.*, 2002). Some mutants were found, which lead either to the (*R*)- or (*S*)-enantiomeric products of alkane hydroxylation (Peters *et al.*, 2003). Using error-prone PCR, some mutants of P450cam (Joo *et al.*, 1999) and P450 BM-3 (Cirino and Arnold, 2003) were created, which demonstrated a high activity with hydrogen peroxide, which acts as both an oxidant and electron donor, thereby substituting NAD(P)H (Urlacher *et al.*, 2004). However, the vast majority of error prone PCR-generated fragments are generally lost during the subcloning step, making it the bottleneck in the mutant library construction procedure (Bichet *et al.*, 2004). On the other hand, the advantage of DNA family shuffling in comparison to other directed evolution techniques is the generation of highly diverse variants with a low level of deleterious mutations (Bacher *et al.*, 2002). The DNA shuffling was introduced first in a single gene (Stemmer, 1994 a, b) and was subsequently extended to family shuffling, involving multi-gene shuffling of a limited number of evolutionarily related parental forms with high sequence identity (Crameri *et al.*, 1998). The screening of the shuffled library of three highly homologous mammalian genes (CYP2C9, CYP2C11 and CYP2C19) for indole oxidation revealed some clones with higher levels of indigo pigment production to those of the parental P450 enzymes and two clones with elevated P450 expression (Rosic *et al.*, 2007).

In general, directed mutagenesis involves 2 steps. Firstly, a mutant library is generated and secondly, a mutant enzyme, which is changed or improved regarding desired characteristics, is selected through a screening process (Slazar and Sun, 2003). The most

complicated part of the screening process is to develop and optimize a rapid screening system, which characteristics must be adapted to the studied protein.

1.4.2. Site-directed mutagenesis

Site directed mutagenesis has been used as a tool for molecular modeling, improving substrate range or evolutionary study for cytochromes P450. It was successfully applied to identify residue responsible for substrate specificity in many cytochromes P450 In contrast to the directed evolution, the rational design of an enzyme through site-directed mutagenesis requires a solid structural basis and an understanding of its catalytic mechanism (Urlacher *et al.*, 2004). In recent years, in the absence of crystal structures, homology modeling has become an important tool to study P450 functions. The models can be implemented to identify or confirm key residues, evaluate enzyme-substrate interactions and explain changes in protein stability and/or regio- and stereo-specificity of substrate oxidation upon residue substitution by site-directed mutagenesis. This phenomenon has also been utilized to analyze the binding of inhibitors or activators, as well as alterations in inhibition and activation due to residue replacement (Szklarz and Halpert, 1997). Several approaches of site-directed mutagenesis have been utilized in past several years. Among this, P450 BM-3 (CYP102A1), from *Bacillus megaterium*, is one of the most extensively studied bacterial cytochromes to generate high catalytic activity. The analysis of the structure and the comparison of substrate-bound and substrate-free forms revealed the importance of the residues R47 and Y51 at the entrance of the substrate access channel. The mutation of these residues alters the enzyme's specificity (Carmichael and Wong, 2001; Cowart *et al.*, 2001). However, the substitution of either residue R47 or residue Y51 alone did not lead to an observable effect on the substrate binding affinity and activity (Li and Poulos, 1999). It was also possible to shift the substrate specificity of P450 from C-12 fatty acids to C-10 and C-8 fatty acids using mutant CYP102A1 F87V in the fatty acid pseudosubstrates 10- and 8-*p*-nitrophenoxycarboxylic acid (Lentz *et al.*, 2001). A triple mutant (CYP102A1 A74G/F87V/L188Q) was able to hydroxylate indole (Appel *et al.*, 2001) and also a wide range of other substrates that have virtually no resemblance to fatty acids, which are regarded as the natural substrates of CYP102A1. A key finding in the latter area was the oxidation of polycyclic aromatic hydrocarbons (PAHs) (Carmichael and Wong, 2001; Li *et al.*, 2001). Three amino acid substitutions in CYP102 (R47L/Y51F/A264G) led to a 200-fold increase of the activity of this enzyme to PAHs. The available structure of the

enzyme shows non-productive binding of the substrates with their ω-end distant from the iron in a hydrophobic pocket at one side of the active site. This pocket was filled by larger hydrophobic side-chain amino acid replacing residue A82 (Huang *et al.*, 2007) to improve substrate binding and catalytic efficiency. Moreover, mutants (CYP102A1 V78A/F87A/I263G and CYP102A1 S72Y/V78A/F87A) exhibited an alteration in the regio-selectivity of the subterminal fatty acid hydroxylation towards γ- and δ-positions, which have not been observed in the CYP102A1wild type enzyme (Dietrich *et al.*, 2009).

Molecular modeling, by doing site directed mutagenesis, is a useful tool that can aid in elucidating substrate specificities and to complement site-directed mutagenesis studies in order to identify critical structural features of the P450 superfamily and other enzymes (Lewis and Lake, 1995). Moreover, using a combination of directed evolution and site-directed mutagenesis now becomes a choice for molecular evolution researchers because it increases the chance to obtain a desired mutant with interesting characteristics. For example, CYP102A1 wild type has a low hydroxylation activity for β-ionone (<1 min^{-1}). Substitution of phenylalanine by valine at position 87 led to a more than 100-fold increase in β-ionone hydroxylation activity (115 min^{-1}). The enzyme activity could be further increased by both site-directed and random mutagenesis. The mutant CYP102A1 R47Y/Y51F/F87V, designed by site-directed mutagenesis, and the mutant CYP102A1 A74E/F87V/P386S, obtained after two rounds of error-prone polymerase chain reaction (PCR), exhibited an increase in activity of up to 300-fold compared to the wild type enzyme (Urlacher *et al.*, 2006).

1.5. Steroid 11-hydroxylation

The steroid derivatives are difficult to synthesize by chemical pathways due to low regio-selectivity and reaction rate, whereas the synthesis can be obtained by biotransformation with microorganisms with specific regio-selectivity as well as a higher turnover rate. In 1952, Murray and Peterson from the Upjohn company (Michigan, USA) patented the process of 11α-hydroxylation of progesterone by *Rhizopus* species (Murray and Peterson, 1952). Since then, a wide range of publications concerning the *Rhizopus* species (*R. nigrican*, *R. arrhizus*, *R. stolonifer*) and their 11α-hydroxylation on different substrates is reported (Gabinskaia *et al.*, 1971; Eysymontt *et al.*, 1975; Holland and Chenchaiah, 1985; Breskvar *et al.*, 1987; Choudhary *et al.*, 2005). The 11-hydroxylation of steroids is mainly found in the fungal kingdom. The 11β-hydroxylase of the filamentous fungus

Cochliobolus lunatus hydroxylates 17α,21-dihydroxypregna-1,4-diene-3,20-dione-17-acetate to yield 11β-deoxyprednisolone-17-acetate, leading to form prednisolone 17-acetate and subsequently prednisolone. Prednisolone is useful for the treatment of a wide range of inflammatory and auto-immune conditions such as asthma (Undisz *et al.*, 1992).

The P450 from *Curvularia lunata* (P450lun) produces cortisol from 11-deoxycortisol in cell-free extracts (Suzuki et al., 1993), as well as an 11α-product from the compound 17α-hydroxypregn-4-ene-3,20-dione-21-acetate (cortexolone-21-acetate, RSA) (Wang *et al.*, 2001) in the presence of NADPH and O_2. Likely, an *Absidia* sp. 28 strain, a cosmopolitan and ubiquitous filamentous fungi in nature, is shown to have high activity of 11α-hydroxylation using 16β-methyl-17α,21-dihydroxy-1,4-pregnadiene-3,20-dione as a substrate (Xu *et al*., 2000). Similarly, another filamentous fungi, *Cunninghamella elegans* was also described to perform the steroid conversion towards 11-hydroxylation. Its protoplasts showed 11α-, and 11β-hydroxylating ability of 17α,21-dihydroxypregna-1,4-diene-3,20-dione (Dtugoński *et al*., 1992).

Progesterone hydroxylation at 11α-position by fungi is also reported in *Aspergillus* species. It was found that the different strains of *Aspergillus* species are involved in the oxygenation at C-11 in the steroid skeleton (Fried *et al.*, 1952; Dulaney *et al.*, 1955; Charney and Herzog, 1967; Clegg *et al.*, 1973). One of the most investigated strains, *A. ochraceus* TS (Samanta *et al.*, 1978; Ghosh and Samanta, 1981; Samanta and Ghosh, 1987), can hydroxylate progesterone in high yields (85 - 90% in 30 min) (Jayanthi *et al.*, 1982). The monooxygenase of *A. ochraceus* TS was capable of 11α-hydroxylation of progesterone. This fungal monooxygenase has many features in common with that of mammalian liver microsomes (Samanta and Ghosh, 1987). Some other strains, *A. fumigatus* and *A. niger*, also exhibit high efficiency of 11α-hydroxyprogesterone formation. *A. fumigatus* hydroxylates exogenous progesterone and forms 11α- and 15β-hydroxyprogesterone as major products (Smith *et al.*, 1994), whereas *A. niger* forms 11α- and 6β-hydroxyprogesterone in 65.7% and 35.5% relative yield (Walaa *et al*., 2009). Besides in the fungal kingdom, the steroid hydroxylation in 11α-position was also discovered in bacteria such as *Bacillus cereus* (Mc-Aleer *et al.*, 1958) and *Streptomyces* species (Shirasaka and Tsuruta, 1960).

In case of human, the 11β-hydroxylase (CYP11B1) participates in the adrenal steroidogenesis converting 11-deoxycortisol to cortisol, the main glucocorticoid in humans. On the other hand, CYP11B2 firstly catalyzes the conversion of 11-deoxycorticosterone to corticosterone and then performs 18-hydroxylation and 18-oxidation to lead aldosterone, the most important human mineralocorticoid. The 11-hydroxylase activity is found not only in the mitochondrial fraction of adrenals but also in the human liver microsomal P450 enzymes. For example, CYP3A4 is found to be responsible for testosterone 11β-hydroxylation in the human liver. It has been suggested that this reaction may have the potential either for diagnosis or therapeutic treatment of adrenal insufficiency diseases (Choi *et al.*, 2005).

As steroid 11α-hydroxylation plays a pivotal role in biotechnology, several researches were carried out and patented. It involves the application of *A. ochraceus, A. niger*, *R. stolonifer*, *R. nigricans*, *R. arrhizus* and strains of *Pestelotia* which are used for the conversion of substrates having a purity of less than 97% and at a concentration greater than 10g/L (Wiersma and van der Meijden, 2000 and 2002).

1.6. Application of steroids as therapeutically agents

The pharmaceutical application of steroids has long been practised. Many steroids are interesting pharmaceutical target substances for the production of anti-inflammatory, diuretic, anabolic, contraceptive, ant androgenic, progestational, and antitumor drugs. For instance, histone deacetylases (HDAC) are involved in estrogen receptor (ER)-mediated gene transactivation. The HDAC inhibitors have been shown to inhibit tumor growth, now are used in clinical trials for the treatment of cancer (Secrist *et al.*, 2003; Bicaku *et al.*, 2008). As the liver X receptors (LXRα and LXRβ) are involved in cholesterol transport, glucose metabolism and inflammation, synthetic LXR ligands have been designed to treat disorders such as atherosclerosis and diabetes (Michael *et al.*, 2005). Some cholestanes are of great interest since they show an antimicrobial effect (Maltais *et al*., 2004; Hanson, 2006).

Glucocorticoids are used in the treatment of inflammatory, kidney, lung, skin, eye and liver diseases, of allergic reactions, blood illnesses, tumors, and gastrointestinal, endocrine and neurological diseases (Buckingham, 2006). Estrogens may be useful for the treatment and prevention or delay of the onset of Alzheimer's disease (Honjo *et al*.,

2003). Progesterone is applied in combined oral contraceptives. It is helpful for women not only who require contraception but also who expect the advantage of avoiding seborrhea or acne (Raudrant and Rabe, 2003; Sitruk-Ware, 2008).

Progestins and their derivatives are used mainly as contraceptives and in hormone replacement therapy for women in the menopause. The 17α-hydroxyprogesterone derivative is clinically applied for the treatment of breast cancer (Pasqualini *et al*., 1998). Some other mono-hydroxyprogesterones, e.g. 11α- and 11β-hydroxyprogesterone, have been reported to inhibit 11β-hydroxysteroid dehydrogenase, which plays an important role of blood pressure regulation (Souness and Morris, 1996; Morita *el al.,* 1996). 11α-hydroxyprogesterone also exhibits an anti-androgenic activity with minimal estrogenic and progestational side effects (Tamm *et al.*, 1982; van der Willingen *et al.,* 1987). 9α-hydroxyprogesterone is a useful intermediate in the production of fludrocortisone and fluoxymesterone, which show both glucocorticoidal and progestational activity (Kang *et al.,* 2004; Lisurek *et al.,* 2004). Dienogest and drospirenone have been synthesized in the last decade and may be considered as the fourth-generation of progestins. They have instead of an androgenic effect a partial anti-androgenic effect which leads to decreased salt and water retention and a lowering in blood pressure (Sitruk-Ware, 2008).

Taken together, it can be stated that steroids are of high pharmaceutical and thus biotechnological value. Cytochromes P450 are involved in modifying steroid molecules and are therefore target for site-directed mutagenesis and directed evolution to improve their properties for a potential industrial application.

1.7. Aim of the work

The overall purpose of this work is to create of mutants CYP106A2 from *Bacillus megaterium* ATCC 13368 by site-directed mutagenesis, which hydroxylate progesterone mainly in 11α-position in comparison with the published 15β-position. The shift of hydroxylation from the 15β- to 11α-position will help to understand more details for the study of structure-function relationship of this enzyme. Moreover, the aim is also focused to produce 11α-hydroxyprogesterone due to its higher pharmaceutical value as compared with the application of the 15β-hydroxyprogesterone. The main objectives of my dissertation are as follows:

1) To select mutant clones from the saturation mutagenesis library at position A395 and G397, which lie on the putative substrate recognition site (SRS) 6 of CYP106A2, showing increased production of 11α-hydroxyprogesterone.

2) To create mutants by site directed mutagenesis which show a high catalytic efficiency with retaining their regio-specificity towards 11α-hydroxyprogesterone.

3) To compare the *in vivo* and *in vitro* progesterone conversion for the selected mutants.

2. MATERIALS AND METHODS

2.1. Materials

All the chemicals and reagents used in this work have analytical grade. The steroid standards (20β-, 15β-, 11α-, 9α-, 6β-hydroxyprogesterone), are gifts from AG Schering company (Berlin, Germany).

2.1.1. Bacteria

The *Escherichia coli* bacterial strains used in this work are summarized in the Table 2.1.

Table 2.1: Genotype of the *E. coli* strains applied in the experiments.

Strain	Genotype	Reference	Comment
TOP10F'	F'{*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	US Patent 5,487,993 (Invitrogen, 2004)	used for minipreparation
JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17(r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F' *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]	Yanisch-Perron et al. (Promega, 1985)	expression of CYP106A2

2.1.2. Plasmids

In this work, two plasmids pACYC_FHH2 and pBar_Twin were used. Vector pACYC_FHH2 carries cDNA of CYP106A2 and pBar_Twin carries cDNAs of adrenodoxin reductase (AdR) and of truncated adrenodoxin (Adx 4 - 108) (Hannemann *et al.*, 2006). These plasmids were coexpressed in *E.coli* JM109 to be able to screen for active mutants as well as for studying the *in vivo* progesterone hydroxylation.

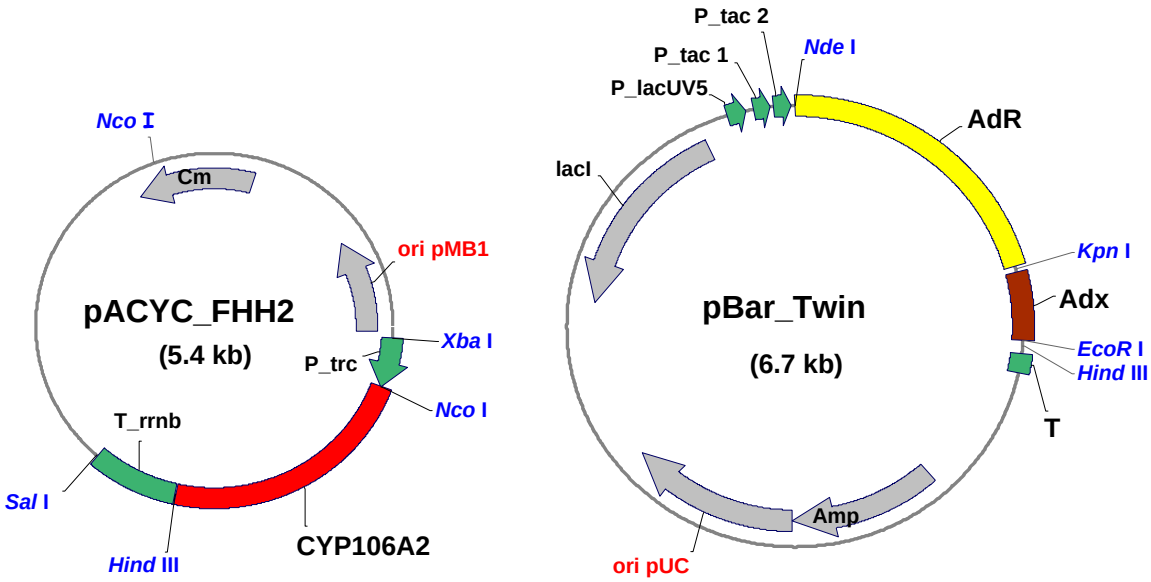


Figure 2.1: CYP106A2 is encoded on the 5.4 kb construct pACYC_FHH2 under the control of tac promoter and a transcription terminator derived from the rrnB gene. The plasmid features the low-copy pMB1 origin of replication and the sequence of the "*cat*" resistance gene. The 6.7 kb vector pBAR_Twin contains two copies of the tac and one lacUV5 promoter, the cDNAs from AdR and Adx, the β-lactamase gene, and the high-copy origin of replication of the pUC series of plasmid (Hannemann *et al.*, 2006).

2.1.3. Oligonucleotides

Oligonucleotides were synthesized by Eurofins MWG Synthesis Company (Ebersberg, Germany). The primers for site directed mutagenesis were designed with an online tool to introduce a silent site selector into the primer (http://rana.lbl.gov/SSS/bin/SSS2.cgi). The sequences of the oligonucleotides were presented in Table 2.2.

Table 2.2: Sequences of the oligonucleotides (*part 1*).

Name	Target	Sequence (5'→ 3')
F2for	Forward primer for sequencing the whole vector pACYC_FHH2_CYP106A2	GATCTCTAGACTGCACGGT GCACCAATGC
F2rev	Reverse primer for sequencing the whole vector pACYC_FHH2_CYP106A2	TGCGGTCGACAAAAAGAG TTTGTAGAAACGC
T89Nfor	Forward primer for Quik Change at position T89N containing the restriction site for *Ase* I	CCTGAAAAGATCCAAATT AATGAATCGGATCCACCT
T89Nrev	Reverse primer for Quik Change at position T89N containing the restriction site for *Ase* I	AGGTGGATCCGATTCATTA ATTTGGATCTTTTCAGG
A106Tfor	Forward primer for Quik Change at position A106T containing the restriction site for *Acl* I	CTGGCAGCAACGTTCACA CCT
A106Trev	Reverse primer for Quik Change at position A106T containing the restriction site for *Acl* I	AGGTGTGAACGTTGCTGC CAG
D217Vfor	Forward primer for Quik Change at position D217V containing the restriction site for *Sca* I	GCGGATGATATCATCTCA GTACTATTGAAGTCGG
D217Vrev	Reverse primer for Quik Change at position D217V containing the restriction site for *Sca* I	CCGACTTCAATAGTACTGA GATGATATCATCCGC
A243Vfor	Forward primer for Quik Change at position A243V	ATTTTAGGTGTAGGAGTCG AG
A243Vrev	Reverse primer for Quik Change at position A243V	CTCGACTCCTACACCTAAA AT
R409Lfor	Forward primer for Quik Change at position R409L containing the restriction site for *Nhe* I	CCGCTTAAGGCTAGCCTCA TGTAA
R409Lrev	Reverse primer for Quik Change at position R409L containing the restriction site for *Nhe* I	TTACATGAGGCTAGCCTTA AGCGG

Table 2.2: Sequences of the oligonucleotides (*part 2*).

Name	Target	Sequence (5'→ 3')
F165Lfor	Forward primer for Quik Change at position F165L containing the restriction site for *Bsm* BI	CGAAAGATCGTCTCTTGTT GAAGAAATGGGTGG
F165Lrev	Reverse primer for Quik Change at position F165L containing the restriction site for *Bsm* BI	CCACCCATTTCTTCAACAA GAGACGATCTTTCG
T247Afor	Forward primer for Quik Change at position T247A containing the restriction site for *Spe* I	GGAGTCGAGGCAACTAGT CAT
T247Arev	Reverse primer for Quik Change at position T247A containing the restriction site for *Spe* I	ATGACTAGTTGCCTCGACT CC
T247Vfor	Forward primer for Quik Change at position T247V containing the restriction site for *Spe* I	GGTGCAGGAGTCGAGGTA ACTAGTCATTTATTGGCC
T247Vrev	Reverse primer for Quik Change at position T247V containing the restriction site for *Spe* I	GGCCAATAAATGACTAGT TACCTCGACTCCTGCACC
T247Ffor	Forward primer for Quik Change at position T247F containing the restriction site for *Spe* I	GGTGCAGGAGTCGAGTTT ACTAGTCATTTATTGGCC
T247Frev	Reverse primer for Quik Change at position T247F containing the restriction site for *Spe* I	GGCCAATAAATGACTAGT AAACTCGACTCCTGCACC
T247Sfor	Forward primer for Quik Change at position T247S containing the restriction site for *Spe* I	GGAGTCGAGTGGACTAGT CAT
T247Srev	Reverse primer for Quik Change at position T247S containing the restriction site for *Spe* I	ATGACTAGTCCACTCGACT CC
T247Wfor	Forward primer for Quik Change at position T247W containing the restriction site for *Spe* I	GGTGCAGGAGTCGAGTGG ACTAGTCATTTATTGGCC
T247Wrev	Forward primer for Quik Change at position T247W containing the restriction site for *Spe* I	GGCCAATAAATGACTAGT CCACTCGACTCCTGCACC
T248Afor	Forward primer for Quik Change at position T248A containing the restriction site for *Ahd* I	GCAGGAGTCGAAACAGCC AGTCATTTATTG

Table 2.2: Sequences of the oligonucleotides (*part 3*).

Name	Target	Sequence (5'→ 3')
T248Arev	Reverse primer for Quik Change at position T248A containing the restriction site for *Ahd* I	CAATAAATGACTGGCTGTT TCGACTCCTGC
T248Vfor	Forward primer for Quik Change at position T248V containing the restriction site for *Ahd* I	GGTGCAGGAGTCGAGACA GTCAGCCATTTATTGGCC
T248Vrev	Reverse primer for Quik Change at position T248V containing the restriction site for *Ahd* I	GGCCAATAAATGGCTGAC TGTCTCGACTCCTGCACC
A395Ifor	Forward primer for Quik Change at position A395I containing the restriction site for *Bmr* I	GAATCTTACCGATTCAATT ACTGGGCAAACTTTG
A395Irev	Forward primer for Quik Change at position A395I containing the restriction site for *Bmr* I	CAAAGTTTGCCCAGTAATT GAATCGGTAAGATTC
A395W/ G397Kfor	Forward primer for Quik Change at positions A395W and G397K containing the restriction site for *Pvu* II	CTTACCGACAGCTGGACC AAGCAAACTTTGACC
A395W/ G397Krev	Reverse primer for Quik Change at positions A395W and G397K containing the restriction site for *Pvu* II	GGTCAAAGTTTGCTTGGTC CAGCTGTCGGTAAG

2.2. Methods

2.2.1. Preparation of *E.coli* competent cells

The preparation of chemically and electro-competent *E.coli* cells TOP10F' and JM109 was carried out as described by Sambrook and Russell (Sambrook and Russell, 2001). The transformation efficiency of the competent cells was checked.

2.2.1.1. Chemically competent cells

Bacterial cells were streaked from frozen glycerol stock, plated onto LB agar and incubated overnight at 37°C. Five ml LB medium (Appendix 6.1.1.) with a selected single *E.coli* colony was inoculated and incubated overnight at 37°C with a shaking speed of 180 rpm. The culture was 50-fold diluted in 100 ml fresh LB medium, 500 µl of 2M $MgSO_4$ was added and shaken continuously at 37°C until an OD_{600} of 0.4 - 0.6 (approximately 2.5 - 3 hr) was reached. The cells were harvested in sterile 50 ml Falcon

tubes at 3500 rpm for 5 min. The supernatant was discarded and the pellet was gently resuspended in approximate 8.5 ml of transfer buffer I (TFPI) (Appendix 6.2.1.1.). The cells were stored on ice for 1 hr. After the second centrifugation at 3500 rpm for 5 min, the cells were gently resuspended in approximately 1 ml of transfer buffer II (TFPII) (Appendix 6.2.1.2.). Aliquots of 50 µl volume of the competent cells were stored at minus 80°C.

2.2.1.2. Electro-competent cells

Preferably, a single colony of *E.coli* from fresh LB plate was selected for inoculating a 10 ml LB overnight starter culture in a 37°C shaker. Subsequently, the culture was diluted 100-fold in 1 L fresh LB medium and placed in a 37°C shaker until the OD_{600} reached 0.4 - 0.6. The culture was removed from the shaker and placed on ice. The cells were centrifugated at 4000 rpm, and 4°C for 15 min. The supernatant was discarded immediately and the pellet was gently washed twice in ice cold destillated H_2O and one more time in 20 ml ice-cold 10% glycerol. The pellet was centrifuged at 4000 rpm, 4°C for 10 min and after removing the supernatant, each pellet was resuspended in 1ml ice-cold 10% glycerol. 50 µl aliquots of cells were frozen in liquid nitrogen and stored at minus 80°C.

2.2.2. Transformation of the plasmid

With the aim to introduce the plasmid DNA into *E.coli* cells, heat shock transformation and electroporation were applied. The stressed cells (by physical factors) were recovered by nutrient SOC medium (Appendix 6.1.4.) through shaking at 37°C for 1 hr.

2.2.2.1. Heat shock transformation

Approximately, 20 ng plasmid DNA was added into a 50 µl freshly thawed aliquot of competent cells and placed on ice for 20 min. The heat shock was performed at 42°C for 90 sec followed by incubation on ice for 5 min. Afterwards, 1 ml SOC medium was added to the cell tube and shaken at 37°C, 200 rpm for 1 hr. Suspensions (200 µl) were plated out on LB agar containing appropriate antibiotic.

2.2.2.2. Eletroporation

To electroporate DNA into the cells, the electrocompetent cells were mixed with the plasmid DNA to be transformed and then pipetted into a 0.1 cm plastic cuvette containing electrodes (Peqlab, Erlangen, Germany). A short electric pulse, 1800 V/cm, was applied to the cells causing smalls holes in the membrane through which the DNA entered. The cells were then incubated with SOC medium before plating out on LB agar containing appropriate antibiotic.

2.2.3. Preparation of plasmid

2.2.3.1. Minipreparation

Plasmid rescue and purification were performed following the instruction of a commercially available kit from Macherey-Nagel (Nucleobond® Minipreparation, http://www.mn-net.com). The DNA yield and purity were determined using the absorbance at 260 nm where DNA absorbs light most strongly. DNA concentration can be estimated by the following equation:

$$\text{Concentration } (\mu g/ml) = A_{260} \times f \times x\varepsilon$$

A_{260}: absorbance at 260 nm

f: dilution factor

$x\varepsilon$: A_{260} of the pure double strands DNA = 50 μg/ml

2.2.3.2. "Quick and dirty" preparation

A transformed *E.coli* culture was incubated in 5 ml LB plus chloramphenicol (35 μg/ml) at 37°C overnight. The culture was centrifuged at 10000 rpm for 5 min. The pellet was resuspended in 0.3 ml of L1 buffer (Appendix 6.2.2.). L2 buffer (Appendix 6.2.2.) was added with a volume of 0.3 ml, mixed gently and incubated for 5 min at room temperature. 0.3 ml L3 buffer (Appendix 6.2.2.) was added and mixed. The suspension was centrifuged at 10000 rpm for 1 min for phase separation. Afterwards the supernatant was transferred and extracted with 450 μl PCIA (Phenol/Chloroform/Isoamylalcohol, 25/24/1) and then 450 μl CIA (Chloroform/Isoamylalcohol, 24/1) for a second time. The DNA plasmid was precipitated by adding 750 μl isopropanol, mixed and centrifuged at

15000 rpm and 4°C for 30 min. The DNA pellet was washed once with 500 µl of 70% ethanol and dried by Speed-vac (Univapo 100H, UniEquip, München, Deutschland). The plasmid was dissolved in 50 µl distillated water.

2.2.4. Separation of plasmids pACYC_FHH2 and pBAR_Twin

For screening, a cotransformation of *E.coli* with plasmids pACYC_FHH2 (containing the cDNA of CYP106A2 wild type or mutants and a chloramphenicol resistance gene) and pBAR_Twin (containing the cDNA of adrenodoxin, the cDNA of adrenodoxin reductase and an ampicillin resistance gene) (Figure 2.1) was required. After screening, these plasmids had to be isolated. The cell-material from glycerol stock (-80°C) was inoculated in 5 ml LB plus 35 µg/ml chloramphenicol and incubated at 37°C overnight. Subsequently, the plasmid was isolated via the "Quick and Dirty" preparation and transformed into chemical competent *E.coli* cells JM109 by heat shock. The cells were spread out on LB agar containing chloramphenicol and incubated overnight at 37°C. Approximate 10 single colonies were picked from the LB agar plate and transferred to another LB agar containing only chloramphenicol (35 µg/ml) as well as to LB agar containing chloramphenicol (35 µg/ml) and ampicillin (100 µg/ml). The plates were incubated overnight at 37°C. Only clones containing pACYC_FHH2_CYP106A2 were able to grow on chloramphenicol. These clones then were inoculated in LB medium plus chloramphenicol for minipreparation.

2.2.5. Site-directed mutagenesis

Site-directed mutagenesis was used to make specific point mutations, and delete or insert single or multiple amino acids. In this method, *Pfu* DNA polymerase was used to replicate both plasmid strands with a high fidelity. The procedure was performed using a recombinant vector with an inserted gene of interest and 2 oligonucleotide primers containing the desired mutation. This incorporation of primers generated a mutant plasmid containing staggered nicks. The treatment of the product with *Dpn* I resulted in the digestion of the parental DNA template.

All the mutations in cDNA of CYP106A2 were generated in the vector pACYC_FHH2 by site-directed mutagenesis using Quik-Change Site-Directed Mutagenesis Kit (Stratagene Ltd, Cambridge, UK) according to manufacturer's instruction and using

mutagenic primers list under section 2.1.3. The reaction was carried out as shown in Table 2.3.

Table 2. 3: PCR reaction mix for plasmid DNA.

Component	Volume
10xP*fu* reaction buffer	5 µl
dNTPs (100 mM)	2 µl
plasmid (10 ng)	1 µl
primer forward (10 pmol)	1 µl
primer reverse (10 pmol)	1 µl
Pfu polymerase	1 µl
water (dd H_2O)	up to 50 µl

Reaction was run in the Thermal cycler PT-100 (MJ Reasearch Inc.) with the following program:

95°C for 5 min
95°C for 30 sec
60°C for 1 min } 18 cycles
72°C for 10 min
72°C for 10 min
4°C for ever

The PCR product was digested with 1 µl of *Dpn* I (10 U/µl) for 3 hr at 37°C to remove the parental template. To increase the efficiency of PCR product transformation by reducing the salt concentration, 20 µl of PCR product was dialyzed into a petri plate containing 10% glycerol through the filter membrane (Millipore, Typ VS 0.025 µm, ∅ 25mm) for 1 hr at room temperature. After dialyzing, all the 20 µl PCR product was used for electroporation into competent *E.coli* TOP10F' and spread out on the LB plate containing chloramphenicol. On the next day, the single colonies (approximate 6 clones) were inoculated in 5 ml of the LB plus chloramphenicol and shaken overnight for plasmid minipreparation. Each clone was checked with corresponding restriction digestion and the positive clones were sent for sequencing to confirm the mutation.

2.2.6. Sequencing and analyzing mutagenesis

The pACYC_FHH2_ CYP106A2 mutant plasmids were sent to Eurofins MWG Company (Ebersberg, Germany) for sequencing. The alignments of sequences between CYP106A2 wild type and mutants were performed using the program CLUSTALW 1.8. (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Translations of amino acids were carried out by using the Translate tool (Gasteiger *et al.*, 2003) and the software available at http://www.expasy.org/tools/dna.html .

2.2.7. Heterologous expression of CYP106A2

CYP106A2 wild type and mutant plasmids were transformed into chemically competent *E.coli* cells JM109 and inoculated in LB medium containing chloramphenicol at 37^{o}C overnight for obtaining the preculture (section 2.2.2.1.). Main culture was prepared in 2 L Erlenmayer flasks containing 1L TB medium (Appendix 6.1.2.) and antibiotics. The preculture was transferred into the main culture (1/100 v/v) and inoculated at 37^{o}C until OD_{600} reached 0.8 - 1.0. Expression procedure was carried out at 30^{o}C with shaking speed of 200 rpm for 48 hr, after adding 1 mM IPTG and 0.5 mM 5-aminolevulinic acid. The cells were harvested by centrifugation at 6000 rpm for 15 min and stored in –20^{o}C for the next study.

2.2.8. Purification of CYP106A2

All the purification steps were carried out at 4^{o}C. After thawing of the pellet from minus 20^{o}C, the cells were suspended in Lysis buffer (Appendix 6.2.3.) and then disrupted by sonication (USD 30, WV) for 20 min on ice (Amplitude 30 μm, Puls 30 s, Ratio 1). Subsequently, the cells were ultracentrifuged at 35000 rpm for 20 min. The supernatant was precipitated in 30 - 70% ammonium sulphate slowly. After ultracentrifugation, the pellet was suspended in 100 ml Buffer A (Appendix 6.2.3.). The protein CYP106A2 was concentrated by a Centriprep-YM30 device (Millipore, Bedford, MA, USA) and diluted with distilled water 1/4 before applying onto a diethylaminoethy anion exchange column (DEAE-Sepharose, Tosohass, Stuttgart, Germany). The protein was washed with Buffer A to reduce salt and non-specific proteins and then eluted by Buffer B (Appendix 6.2.3.) with flow rate 1.5 ml/min and following gradient:

0 - 50 ml:	100% Buffer A
50 - 130 ml:	35% Buffer B
3.0 ml/fraction	
130 - 250 ml:	50% Buffer B
250 - 300 ml:	100% Buffer B

Only CYP106A2 fractions having $A_{417/280} \geq 0.6$, were collected, diluted with distilled water (1/1 v/v) and then applied onto a *Macro prep ceramic* hydroxy apatite (HAP) column (BioRad, Hercules, CA, USA). Buffer C (Appendix 6.2.3.) was used for equilibration and washing the column. The elution was carried out at a flow rate of 1.5 ml/min with Buffer D (Appendix 6.2.3.). Following gradient system was used:

0 - 30 ml:	100% Buffer C
30 - 120 ml:	40% Buffer D
4.5 ml/fraction	
120 - 230 ml:	80% Buffer D
230 - 300 ml:	100% Buffer D

The collected fractions, which had $A_{417/280} \geq 0.8$ after concentration by Centriprep-YM30, were finally applied onto Gel filtration using a Superdex 75 column (Amersham Pharmacia Biotech). Buffer E (Appendix 6.2.3.) was used for washing and to elute the protein with a flow rate of 0.3 ml/min and 1 ml/fraction. The purified protein fractions having $A_{417/280} \geq 1.0$ - 1.2 were concentrated by Centriprep YM30 until a total volume of 500 µl was reached.

2.2.9. UV-Vis and CO-difference spectra measurements

The purified CYP106A2 was measured using an UV-Vis spectrometer of Shimadzu Photometer (UV2101PC, Kyoto, Japan) at wavelength from 200 - 700 nm.

Carbon monoxide (CO) different spectra were used to quantify the cytochrome P450 concentration according to Omura and Sato (Omura and Sato, 1964). Cytochromes P450 exhibit a Soret peak at 450 nm in their reduced form iron-CO complex.

The concentration was calculated according to the Beer–Lambert equation:

$$c(\text{P450}) = \frac{\Delta A_{450-490} \times \text{dilution factor}}{\varepsilon \times d}$$

c(P450)	concentration of cytochrome P450 [mM]
$\Delta A_{450-490}$	absorptional difference at 450 nm and 490 nm
ε	molar extinction coefficient [91 $mM^{-1} \times cm^{-1}$]
d	diameter or length of the cuvette [cm]

2.2.10. Determination of protein concentration (BC Assay)

Bicinchoninic acid (BC) assay protein quantitation kit (Uptima Interchim, Montluçon, France) was used for protein concentration measurement. The BC assay is a colorimetric assay, which involves the reduction of Cu^{2+} to Cu^{+} by proteins in an alkaline medium and chelates Cu^{+} ions with very high specificity to form a water soluble purple colored complex.

The reaction was measured after 30 min at 37°C by using the high optical absorbance of the final Cu^{+} complex at 562 nm. Absorbance was directly proportional to the protein concentration, with a broad linear range between 20 - 2000 μg/ml. Protein concentration was calculated with a reference curve obtained for a standard protein which was obtained from bovine serum albumin (BSA). Standard protein solutions were prepared freshly ranging from 20 μg/ml to 2000 μg/ml and were diluted from the stock solution in the same buffer (Appendix 6.3.).

2.2.11. Sodium dodecylsulphate (SDS) polyacrylamid gelelectrophoresis

Separation of proteins according to their molecular mass was conducted using the Laemmli discontinuous gel electrophoresis (SDS-PAGE) method (Laemmli, 1970). For preparing the gel, the separating gel solution (Appendix 6.4.1.) was carefully poured between glass plates, overlaid with distilled water and allowed to polymerize at room temperature for 30 min. After removing the overlaying water, the stacking gel solution (Appendix 6.4.2.) was poured and a comb was inserted. The gel was allowed to polymerize for further 30 min. The gel was stored in soaked papers with water at 4°C until use.

A portion of the protein sample was mixed with 2 x SDS loading buffer (1/1 v/v) and heated at 100^{o}C for 5 min. The samples were applied to the slots of the stacking gel for 15 min at 80 V and then separated on a separating gel at 100 V until the bromophenol blue front reached the bottom of the gel.

After running SDS-PAGE, the gel was stained with coomasie staining solution for 1 hr at room temperature and then incubated in destaining solution until the bands for the proteins were clearly visible. The gel was finally dried on a gel dryer (Model 583 gel dryer, BioRad) for storage.

2.2.12. Reconstitution of *in vitro* steroid conversion

Enzyme activity towards progesterone conversion was performed in 50 mM HEPES buffer, pH 7.4. The reconstitution system was established in a total volume of 500 µl using a NADPH regeneration system (5 mM glucose-6-phosphate, 1 U glucose-6-phosphate dehydrogenase, 1 mM $MgCl_2$ and 0.1 mM NADPH). The concentration of Adx (4 - 108) was 10 µM, of AdR 0.5 µM and of CYP106A2 from wild type and mutants was constant, 0.25µM. Progesterone concentration was varied from 17.5 - 600 µM. The reaction was carried out in 30^{o}C for 10 min at 12000 rpm and stopped by addition of 500 µl chloroform. The steroids were extracted twice with chloroform and evaporated using Speed-Vac (Univapo 100H, UniEquip, Munich). The residues were resolved with acetonitril for HPLC analysis.

2.2.13. Screening of mutants

This method allows to screen the mutants faster which is interesting for steroid conversion based on the colorimetric assay protocol from Appel *et al.* (Appel *et al*, 2005).

Electro-competent JM109 cells were cotransformed using the plasmids pACYC_FHH2 containing sequences of CYP106A2 wild type and mutants, and with pBAR_Twin containing the sequences of AdR and Adx (4 - 108). Aliquotes (50 µl) of the transformation preparation were spread out on agar plates containing chloramphenicol (35 µg/ml) and ampicillin (100 µg/ml). The plates were incubated at 37^{o}C overnight. A single colonie from each plate was taken and transferred by sterile toothpicks into “half deep well plate” (PP-Masterblock, maximal volume 0.5ml, sterile, Boden V-Form, Greiner Bio-One, Frickenhausen, Germany) containing 350 µl of LB and both of antibiotics. For

each mutant, this procedure was repeated 3 times except for mutants CYP106A2 A106T/A395I/R409L, CYP106A2 A106T/A395W/G397K/R409L and CYP106A2 T89N/A106T/A395I/R409L, which were repeated twice. The last three positions in the plate were used for inoculation of CYP106A2 wild type. The microtiter plate was sealed with a membrane (Breathseal, Greiner, Germany) and shaken at 37^{o}C, 300 rpm overnight (Innova Shaker 4230, New Brunswick Scientific, Nürtingen, Germany). On the next day, 45 µl of preculture was transferred into main culture prepared in "deep well plate" (Mega Block 2.2ml, Sarstedt, Nürnbrecht, Germany) containing 1ml of TB media with 0.5 mM of 5-aminolevulinic acid, 1 mM of IPTG, 140 µl/L of trace element solution (Appendix 6.1.3.) as well as 400 µM of progesterone and both antibiotics. The "deep well plate" was sealed and inoculated at 30^{o}C, 300 rpm for 48 hr. Afterwards the cultures were harvested by centrifugation at 3000 rpm for 20 min. A volume of 600 µl of the supernatants were transferred into another clean "deep well plate". Subsequently, 300 µl of methyl isobutyl ketone (Sigma-Aldrich, Seelze, Germany) was added for extraction of the steroids. After separation in 2 phases by centrifugation at 3000 rpm for 10 min, 100 µl of the upper phase was transferred into a black acid resistant polypropylene microtiter plate with F-trays (Greiner, Germany). The organic phase then was mixed with 150 µl of a sulfuric acid/acetic acid (90/10 v/v). Fluorescence was measured after incubation in darkness for 10 min by Plate reader Genios (Tecan, Crailsheim, Germany) and using the Xflour 4 software. The measurement was set to following conditions:

Excitation wavelength:	485 nm
Emission wavelength:	535 nm
Gain:	optimal
Integration time:	20 µs
Number of flashes:	5

The "gain" is the parameter to control sensitivity in the measurement of the process. The average of all the measurement was done.

2.2.14. Whole-cell steroid conversion

Cotransformed cells JM109 containing two plasmids (pACYC_FHH2_CYP106A2 and pBAR_Twin) were prepared as above for preculture (section 2.2.13.). Main culture was prepared in 100 ml Erlenmayer flask containing 20 ml of TB media and antibiotics.

Preculture was transferred into main culture (1/100 v/v) and inoculated at 37°C until OD_{600} reached 0.8 - 1.0, then 1 mM of IPTG and 0.5 mM of 5-aminolevulinic acid were added. The expression procedure was carried out at 30°C, for 24 hr with 200 rpm. An aliquot (1 ml) was centrifuged at 14000 rpm for 2 min and washed once with 50 mM of potassium phosphate buffer (pH 8.0). The cells were suspended in 1 ml of buffer and inoculated at 30°C. Progesterone was dissolved in absolute HPLC grade ethanol or 45% hydroxypropyl-β-cyclodextrin. The reaction was initiated by the addition of substrate (100 μM and 200 μM) to the test cell-media (6 hr and 12 hr, respectively). The reaction was stopped by incubation for 1 min at 100°C to inactivate the biocatalyst. The dead-cells were removed by centrifugation at 15000 rpm, 4°C for 5 min. The supernatants were carefully transferred into another clean eppendorf tube for the extraction of steroids by chloroform. The organic phases were transferred and dried by Speed-Vac (Univapo 100H, UniEquip, Munich). The residues were resolved in acetonitril for HPLC analysis.

2.2.15. High Performance Liquid Chromatogram (HPLC)

The steroids were analyzed by using a High Performance Liquid Chromatography system 900er from Jasco Company (Groβ-Umstadt, Germany). The extracted steroids were dissolved in 200 μl of acetonitril before analyzing in the HPLC system. The steroids were detected by an UV-Vis detector at 240 nm, 25°C controlled by an oven (Techlab, Erkerode, Germany) when crossing the column Nova Pak Nucleosil C_{18} (4 μm, 3.9x150 mm) or column Macherey-Nagel CC125/4 Nucleodur 100-5 C_{18}ec. The solvent was a mixture acetonitril/water (40/60) with a flow rate of 1 ml/min. 20β-hydroxyprogesterone was used as internal standard. The data were calculated through hyperbolic equation (f(x) = ax/(b+x)) by using Sigma Plot software (Rockware, Golden, CO, USA).

3. RESULTS

CYP106A2 from *Bacillus megaterium* ATCC 13368 is able to hydroxylate a variety of 3-oxo-4-ene steroids mainly in the 15β-position. Progesterone is converted predominantly into 15β-hydroxyprogesterone and minor amount of 11α-, 9α-, 6β-hydroxyprogesterone (Berg *et al.*, 1976; Lisurek *et al.*, 2004). The minor products range from 5 - 10% relative value compared with 15β-hydroxyprogesterone. The minor products 11α-, 9α- and 6β-hydroxyprogesterone have a higher as pharmaceutical value compared with 15β-hydroxyprogesterone. In 2004, Lisurek constructed and docked progesterone into the model of CYP106A2. Amino acids A395 and T396 of CYP106A2, which lie in the putative substrate recognition site (SRS) 6, revealed a hydrogen bond with progesterone and no other SRS showed an interaction with this substrate. Four residues, S394, A395, T396, G397, in this loop were predicted to have an effect on the regio-selectivity of CYP106A2. Therefore, they were chosen to be mutated. In order to verify the role of these residues, they were altered to corresponding residues of CYP11B1, which resulted in 4 site-directed mutants CYP106A2 S394I, CYP106A2 A395L, CYP106A2 T396R and CYP106A2 G397P. These mutants were expected to change the regio-selectivity of progesterone hydroxylation towards 11α-position. However, the activity of these 4 mutants in progesterone conversion indicated that only 2 positions of CYP106A2 (A395 and G397) seem to play a role in altering the regio-selectivity of hydroxylation to the originally minor products (Lisurek, 2004; Lisurek *et al.*, 2008). Therefore, a saturation mutagenesis library (approximately 13100 transformants) at the positions A395 and G397 was created by Virus using degenerated primers (Virus, 2006).

My work started with randomly selecting 40 transformants from the saturation mutagenesis library of CYP106A2 having mutations at residues 395 and 397. The aim of the work was to screen the mutants for a higher 11α-hydroxyprogesterone production.

3.1. Screening the saturation mutagenesis library of CYP106A2 SRS6 mutants

3.1.1. Separation of plasmids pACYC_FHH2 and pBAR_Twin

Firstly, to know the exact activity of the mutant enzymes in progesterone conversion, each vector pACYC_FHH2 containing a single CYP106A2 mutant from the transformant had to be separated from pBAR_Twin coexpressed *E.coli* JM109. The separation was performed as described in section 2.2.4. The vector pACYC_FHH2 derived from the

original vector pACYC184 was modified by inserting the CYP106A2 expression cassette containing 2 restriction sites *Nco* I and *Hind* III (Hannemann *et al*., 2006).

After minipreparation, the plasmids were checked by restricted digestion with *Nco* I and *Hind* III at 37°C for 4 - 5 hr. The negative control was done without plasmid. Finally, the sizes of the DNA fragments were observed in an 1% agarose gel under UV light. The vector and the inserted DNA yielded a 4.1 kb and 1.2 kb fragment, respectively.

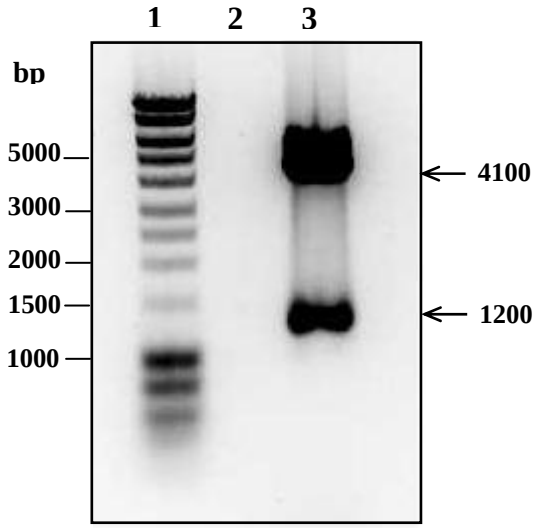


Figure 3.1: Agarose gel electrophoresis of pACYC_FHH2 containing cDNA of CYP106A2 after cutting by restriction enzymes *Nco* I and *Hind* III. 1% agarose gel was used. **1**: Marker, **2**: Negative control, **3**: pACYC_FHH2_CYP106A2 from one mutant.

3.1.2. Heterologous expression of 15β-hydroxylase in *E.coli* JM109

All mutants (containing the single vector pACYC_FHH2_CYP106A2) from the saturation library were transformed and heterologously expressed in chemically competent *E.coli* JM109 cells as described in section 2.2.7. The pellets, obtained after 48 hr expression, were suspended in lysis buffer (Appendix 6.2.3.) and then disrupted by sonification and centrifuged as described in section 2.2.8. Supernatants as “crude extract” enzyme solution were used for CO-difference spectra measurements (Figure 3.2) and the calculated from CYP106A2 concentration is shown in Table 3.1.

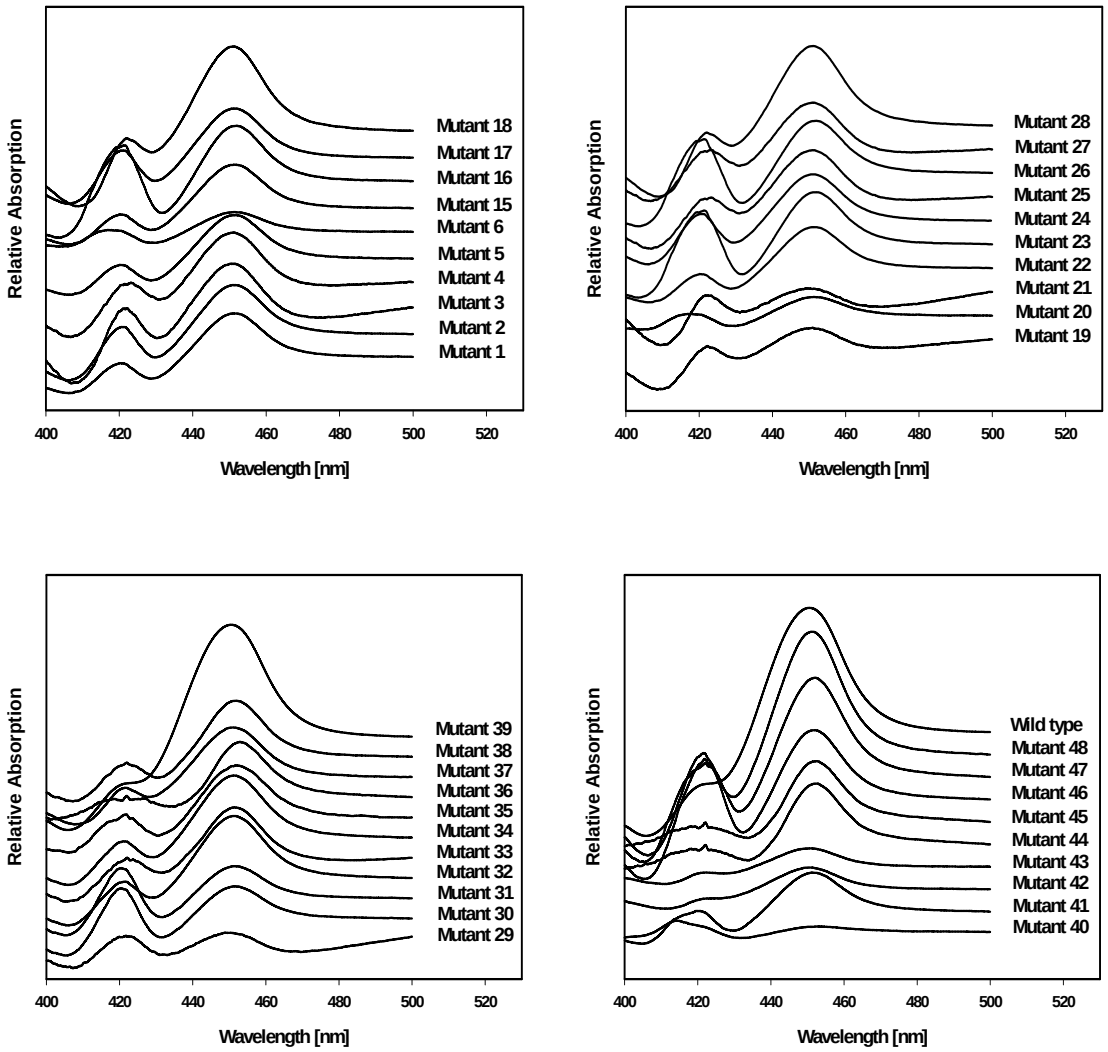


Figure 3.2: CO-difference spectra measurement of the SRS6 CYP106A2 mutants. The "crude extract" enzymes were obtained from the supernatant as after sonification. The measurements were performed following Omura and Sato's method to calculate P450 content (Omura and Sato, 1964).

The CO-different spectra of the mutants showed the typical maximum absorbance at 450 nm. However, a low (or higher) amount of cytochrome P420 in "crude extract" enzymes, which is an inactive form of cytochrome P450 (showing a Soret peak at approximate 420 nm), was also presented.

Table 3.1: Concentration of CYP106A2 mutants and wild type after expression in the "crude extract" enzyme (*part 1*).

Mutant Abbreviation	Concentration of P450 [nmol/L culture]	Position in micro titer plates
Mutant 1	486	291005_01_F8
Mutant 2	511	291005_01_A6
Mutant 3	471	291005_02_F6
Mutant 4	558	211005_02_F7
Mutant 5	536	211005_02_G7
Mutant 6	172	211005_02_D11
Mutant 15	542	211005_02_B9
Mutant 16	691	211005_02_D9
Mutant 17	632	211005_02_C10
Mutant 18	689	211005_02_H11
Mutant 19	201	211005_02_C12
Mutant 20	239	291005_01_H2
Mutant 21	197	291005_01_E5
Mutant 22	490	291005_01_H7
Mutant 23	583	291005_01_A12
Mutant 24	544	291005_02_D3
Mutant 25	537	291005_02_G4
Mutant 26	582	291005_02_D1
Mutant 27	579	291005_02_G6
Mutant 28	681	291005_02_B9
Mutant 29	204	291005_02_D9
Mutant 30	433	291005_02_E11
Mutant 31	423	291005_02_G11
Mutant 32	673	311005_01_A1
Mutant 33	668	311005_01_A5
Mutant 34	671	311005_01_G6
Mutant 35	662	311005_01_H8

Table 3.1: Concentration of CYP106A2 mutants and wild type after expression in the "crude extract" enzyme (*part 2*).

Mutant Abbreviation	Concentration of P450 [nmol/L culture]	Position in micro titer plates
Mutant 36	694	311005_01_E9
Mutant 37	676	311005_01_F9
Mutant 38	673	311005_01_D10
Mutant 39	877	311005_01_F10
Mutant 40	108	311005_02_C3
Mutant 41	574	311005_02_F5
Mutant 42	244	311005_02_C8
Mutant 43	248	311005_02_F1
Mutant 44	654	311005_02_D8
Mutant 45	640	311005_02_E8
Mutant 46	682	311005_02_D10
Mutant 47	885	311005_02_B11
Mutant 48	1194	311005_02_E12
CYP106A2 WT	1273	

The yields of the P450 protein for the mutants varied from 400 to 700 nmol/L culture, and were less than the concentration of CYP106A2 wild type (1273 nmol/L culture). However, some CYP106A2 mutants (6, 19, 20, 21, 29 and 40) showed very low expression levels (approximately 200 nmol/L culture). Only mutant 48 showed a similar expression level as that of the CYP106A2 wild type (1194 nmol/L culture).

In order to supply for *in vitro* conversion of progesterone, all "crude extract" enzymes of CYP106A2 mutants and wild type were purified by applying on the DEAE Sepharose column to reduce the impurities from medium or *E.coli* cells. All CYP106A2 samples were lost approximately 30% - 40% of P450 content after purification (Table 3.2). The ratio A_{417}/A_{280} was used to monitor the purification cytochrome P450 level.

Table 3.2: The yield of cytochrome P450 from CYP106A2 mutants after purification through the DEAE-Sepharose column (*part 1*).

Mutant	Crude extract solution [nmol/L]	After DEAE-Sepharose column [nmol/L]	The ratio A_{417}/A_{280}
Mutant 1	486	361	0.5
Mutant 2	511	342	0.6
Mutant 3	471	303	0.4
Mutant 4	558	398	0.5
Mutant 5	536	411	0.5
Mutant 6	172	120	0.4
Mutant 15	542	387	0.5
Mutant 16	691	442	0.6
Mutant 17	632	418	0.6
Mutant 18	689	426	0.6
Mutant 19	201	171	0.4
Mutant 20	239	182	0.4
Mutant 21	197	129	0.4
Mutant 22	490	372	0.4
Mutant 23	583	426	0.4
Mutant 24	544	377	0.5
Mutant 25	537	361	0.5
Mutant 26	582	384	0.5
Mutant 27	579	367	0.5
Mutant 28	681	434	0.6
Mutant 29	204	148	0.4
Mutant 30	433	366	0.4
Mutant 31	423	370	0.4
Mutant 32	673	457	0.5
Mutant 33	668	432	0.5
Mutant 34	671	469	0.5
Mutant 35	662	472	0.5

Table 3.2: The yield of cytochrome P450 from CYP106A2 mutants and wild type after purification through the DEAE-Sepharose column (*part 2*).

Mutant	Crude extract solution [nmol/L]	After DEAE-Sepharose column [nmol/L]	The ratio A_{417}/A_{280}
Mutant 36	694	484	0.5
Mutant 37	676	492	0.5
Mutant 38	673	441	0.6
Mutant 39	877	579	0.6
Mutant 40	108	67	0.4
Mutant 41	574	401	0.4
Mutant 42	244	172	0.4
Mutant 43	248	168	0.4
Mutant 44	654	451	0.5
Mutant 45	640	469	0.4
Mutant 46	682	482	0.5
Mutant 47	885	531	0.6
Mutant 48	1194	744	0.6
CYP106A2 WT	1273	827	0.6

3.1.3. *In vitro* conversion of progesterone

To verify the enzyme activity of the mutants, after purifying the related proteins, *in vitro* conversions were done as described in section 2.2.12. During conversion the final concentration of the progesterone was 300 μM. The reactions were carried out at 30°C for 10 min and were stopped by adding chloroform. Each activity measurement for a mutant was repeated twice. In the negative control, water was used instead of NADPH. The steroids were extracted twice, evaporated before dissolving in acetonitril and finally applied on a HPLC system 900er (Jasco Company, Germany) using Nova Pak Nucleosil C_{18} (4 μm, 3.9 x 150 mm) column. Products were detected as described in section 2.2.15. The 20β-hydroxyprogesterone was used as an internal standard whereas 15β-, 11α-, 9α- and 6β-hydroxyprogesterone were used as external standards. The retention times of standards are shown in Table 3.3.

Table 3.3: The HPLC retention time of the mono-hydroxylated progesterone product analyzed using Nova Pak Nucleosil C_{18} (4 μm, 3.9 x 150 mm) column with an isocratic solvent of 40% acetonitrile and 60% water with the flow rate of 1 ml/min at 25°C are shown. The steroids were detected at 240 nm.

Mono-hydroxylated progesterone	**Retention time [min]**
20β-hydroxyprogesterone	24.77 ± 0.73
15β-hydroxyprogesterone	3.76 ± 0.03
11α-hydroxyprogesterone	4.14 ± 0.06
9α-hydroxyprogesterone	4.71 ± 0.11
6β-hydroxyprogesterone	5.42 ± 0.09

Most CYP106A2 mutants showed a more diverse hydroxylating pattern of progesterone compared with that of CYP106A2 wild type. During the experiment, some CYP106A2 mutants (1, 4, 15 - 19, 25, 27, 31, 32, 34, 35, 40 and 42) did not show any conversion (Figure 3.3a and 3.3b), whereas other CYP106A2 mutants converted progesterone into 4 main mono- (15β-, 11α-, 9α- and 6β-hydroxylated) products of progesterone along with some di- or poly-hydroxylated products with different amounts. CYP106A2 mutants 41, 46 and 47 (Figure 3.3b) showed the highest amounts of absolute 9α-hydroxyprogesterone formation (22.3 ± 1.2, 36.1 ± 1.2, 21.3 ± 2.6 nmol product/min nmol CYP106A2, respectively) compared with the others.

Concerning 11α-hydroxyprogesterone formation, it can be seen that most mutants gave a higher product yield than CYP106A2 wild type (Figure 3.3a and 3.3b). However, CYP106A2 mutants 2, 3, 37 and 39 showed the highest activity of 18.8 ± 3.0, 19.3 ± 1.2, 20.1 ± 2.8 and 25.7 ± 2.8 nmol product/min nmol CYP106A2, respectively, of 11α-hydroxyprogesterone production.

CYP106A2 mutants 5, 6, 33, 37 and 43 could hydroxylate progesterone at the 6β-position with higher efficiency than the rest of the CYP106A2 mutants and resulted in 13.7 ± 0.8, 16.3 ± 1.3, 15.9 ± 1.5, 19 ± 0.8 and 17.4 ± 2.4 nmol product/min nmol CYP106A2, respectively (Figure 3.3a and 3.3b).

The activity of the CYP106A2 mutants observed during the conversion, is shown in Table 3.4. The progesterone consumption by CYP106A2 wild type for 10 min was set to 100%. In general, none of the CYP106A2 mutants showed a significant faster activity than

CYP106A2 wild type. With 12 CYP106A2 mutants (2, 3, 5, 6, 20, 33, 37, 41, 43, 46, 47 and 48) out of 25 presented as “active mutants”, a relative activity similar to that of CYP106A2 wild type was observed. Some CYP106A2 mutants (21, 24, 29 and 36) showed critically low activities (≤ 25% of CYP106A2 wild type activity) shown in Figure 3.3a, 3.3b and Table 3.4.

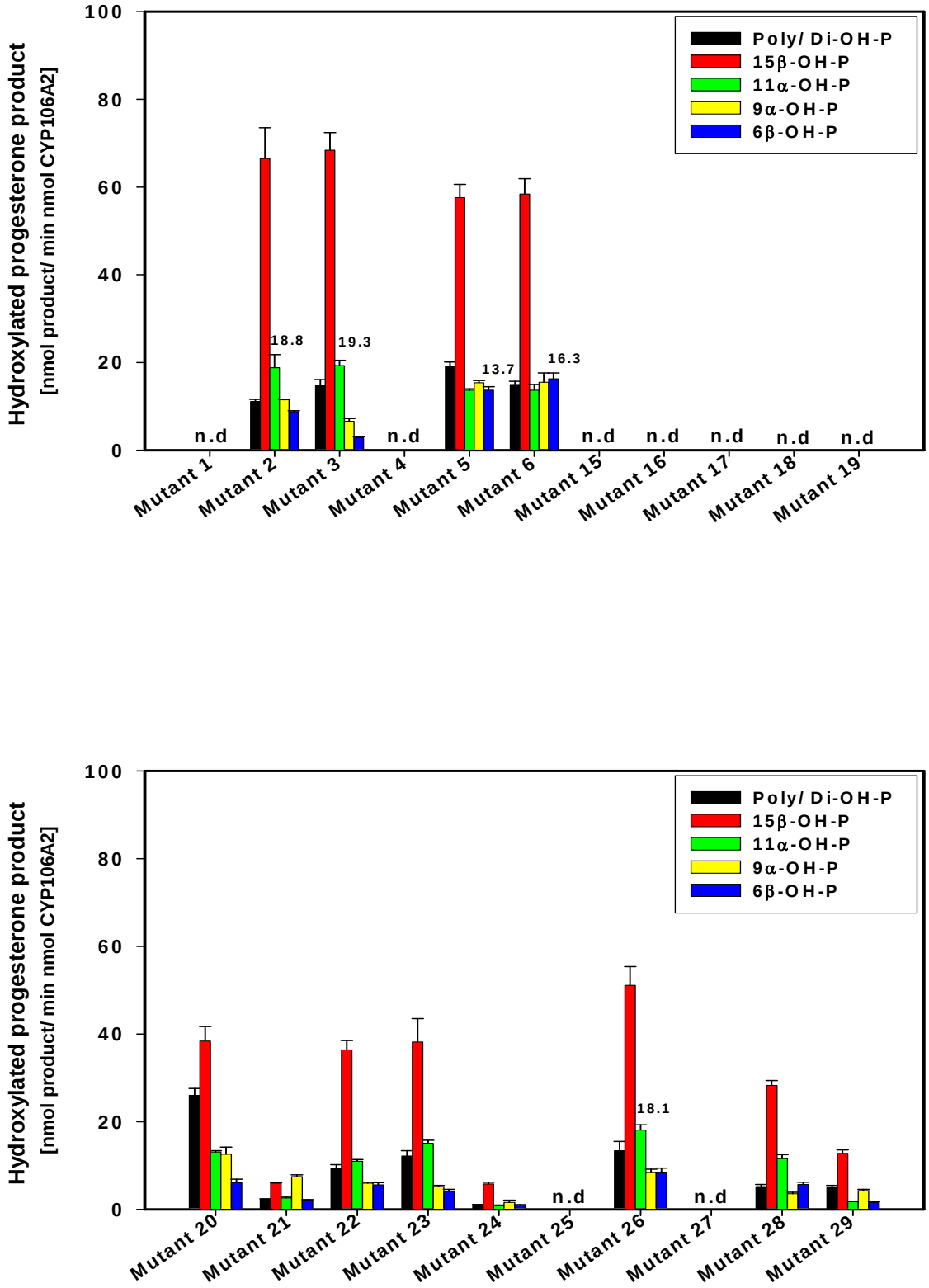


Figure 3.3a: The absolute value of hydroxylated progesterone products for the CYP106A2 mutants 1 to 29. Poly- or di-hydroxyprogesterone product (black), 15β-hydroxyprogesterone (red), 11α-hydroxyprogesterone (green), 9α-hydroxyprogesterone (yellow) and 6β-hydroxyprogesterone (blue) are shown, n.d not detectable.

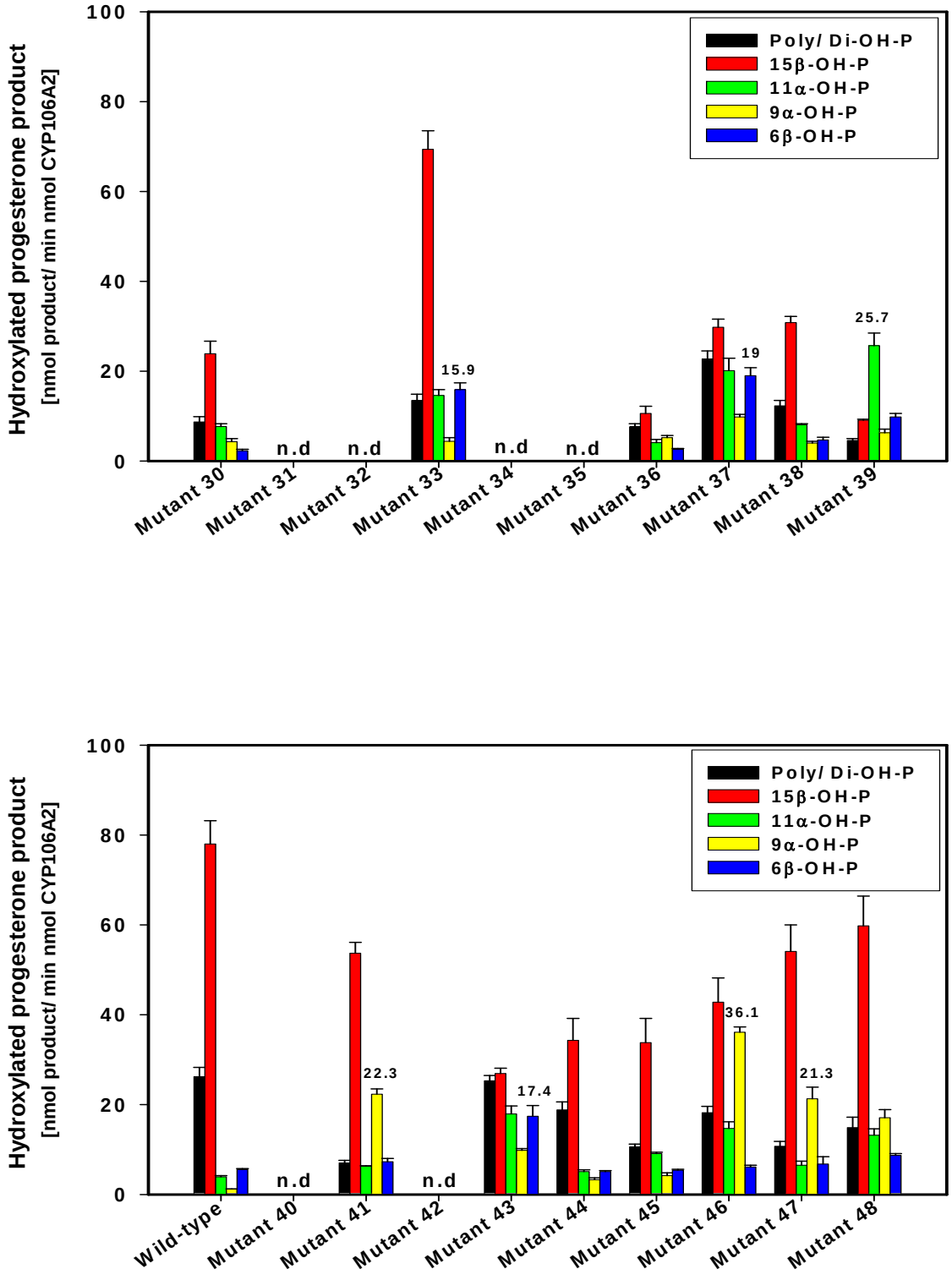


Figure 3.3b: The absolute value of hydroxylated progesterone products from the CYP106A2 wild type and from mutants 30 to 48. Poly- or di-hydroxyprogesterone product (black), 15β-hydroxyprogesterone (red), 11α-hydroxyprogesterone (green), 9α-hydroxyprogesterone (yellow) and 6β-hydroxyprogesterone (blue) are shown, n.d not detectable.

Table 3.4: Comparison of progesterone consumption with CYP106A2 wild type and the mutants.

	Progesterone consumption after 10 min [%]	**Comparison with CYP106A2 wild type**
CYP106A2 WT	95.4	1.00
Mutant 2	96.8	1.01
Mutant 3	93.4	0.98
Mutant 5	99.6	1.04
Mutant 6	99.1	1.04
Mutant 20	80.2	0.84
Mutant 21	17.3	0.18
Mutant 22	56.9	0.59
Mutant 23	62.3	0.65
Mutant 24	8.6	0.09
Mutant 26	82.7	0.87
Mutant 28	45.4	0.48
Mutant 29	21.3	0.22
Mutant 30	39.0	0.41
Mutant 33	98.1	1.03
Mutant 36	25.1	0.26
Mutant 37	84.6	0.89
Mutant 38	49.9	0.52
Mutant 39	46.3	0.49
Mutant 41	80.6	0.84
Mutant 43	81.2	0.85
Mutant 44	55.6	0.58
Mutant 45	52.6	0.55
Mutant 46	98.3	1.03
Mutant 47	82.8	0.87
Mutant 48	94.7	0.99

Besides the actual activities of the mutants, the most interesting part of this analysis was to investigate a possible change of the regio-selectivity of hydroxylation by these mutants. CYP106A2 wild type converts progesterone mainly in 15β-position. On setting of 15β-hydroxyprogesterone to 100%, the minor products of 11α-, 9α- and 6β-hydroxyprogesterone were found to be 5%, 1.5% and 6.4% respectively.

The results shown in Table 3.5 illustrate the differences of the distribution of the products progesterone hydroxylation. All CYP106A2 mutants increased the formation of the minor products by at least 2- or 3-fold. CYP106A2 mutants 21, 36, 39, 41, 46 increased the percentage of the 9α-hydroxyprogesterone from 1.5% to 125.8%, 49.7%, 69.6%, 41.5% and 84.3%, respectively. CYP106A2 mutants 21, 28, 37, 39, 43 also improved the 11α-hydroxyprogesterone formation from 5% up to 43.1%, 41.1%, 67.7%, 282.2% and 66.7%, respectively. CYP106A2 mutants 21, 37, 39, 43 produced the 6β-hydroxyprogesterone up to 36.7%, 63.8%, 107.7% and 64.8%, respectively.

There were 2 cases, CYP106A2 mutants 21 and 39, where the main product was not 15β-hydroxyprogesterone. CYP106A2 mutant 21 produced mainly 9α-hydroxyprogesterone (125%) compared with 15β-hydroxyprogesterone, but unfortunately, the catalytic activity of that mutant enzyme was almost critically 5-fold lower than the wild type protein activity (progesterone consumption after 10 min was 18% compared with protein wild type). Especially, CYP106A2 mutant 39 showed a drastic changed in the regio-selectivity of hydroxylation. The 15β-hydroxyprogesterone became a minor product as compared with other products such as 11α-hydroxyprogesterone and 6β-hydroxyprogesterone (282.2% and 107.7%, respectively). Even though, the activity of the CYP106A2 mutant 39 was half as compared with CYP106A2 wild type. The ratio between 15β-hydroxyprogesterone/11α-hydroxyprogesterone was approximately 1/3. Thus, this mutant showed a considerable shift of the regio-selectivity of hydroxylation from the 15- to the 11-position. This is the greatest shift obtained so far.

Table 3.5: Regio-selectivity of the mono-hydroxyprogesterone products derived from CYP106A2 wild type and mutants. The relative amounts of hydroxylated products are shown. 15β-hydroxyprogesterone was set to 100%.

	15β-OH-P [%]	**11α-OH-P [%]**	**9α-OH-P [%]**	**6β-OH-P [%]**
CYP106A2 WT	100	5.0	1.5	6.4
Mutant 2	100	28.5	17.4	13.3
Mutant 3	100	28.3	9.7	4.2
Mutant 5	100	23.9	26.7	23.8
Mutant 6	100	23.5	26.5	27.8
Mutant 20	100	34.2	32.8	15.8
Mutant 21	100	43.1	125.8	36.7
Mutant 22	100	30.2	16.5	15.3
Mutant 23	100	39.5	13.6	10.8
Mutant 24	100	15.3	28.5	17.0
Mutant 26	100	35.4	16.4	16.2
Mutant 28	100	41.1	12.8	20.0
Mutant 29	100	14.1	33.5	12.6
Mutant 30	100	32.2	17.9	9.5
Mutant 33	100	21.0	6.4	22.9
Mutant 36	100	39.1	49.7	24.7
Mutant 37	100	67.7	33.2	63.8
Mutant 38	100	26.1	13.1	15.1
Mutant 39	100	282.2	69.6	107.7
Mutant 41	100	11.7	41.5	13.6
Mutant 43	100	66.7	36.6	64.8
Mutant 44	100	16.0	9.6	14.9
Mutant 45	100	27.0	12.3	15.8
Mutant 46	100	34.2	84.3	14.2
Mutant 47	100	12.0	39.3	12.6
Mutant 48	100	22.1	28.6	14.6

3.1.4. Sequencing of the active mutants

To evaluate the effect of mutation at positions A395 and G397 of the CYP106A2 on the progesterone conversion, the "active mutants" were sequenced (Eurofins MWG Company, Ebersberg, Germany). The results are described in the Table 3.6.

The obtained results derived from the saturation mutagenesis of CYP106A2 at positions A395 and G397 showed that only in one case, CYP106A2 mutant 39 (CYP106A2 A395W/G397K), a change into an aromatic amino acid group occured. Obviously, this replacement gave a high contribution to the shift in the stereo-selectivity towards 11α-hydroxylation. From the sequencing results of the active mutants, it can be seen that residue A395 has a high frequency of more aliphatic or basic amino acids due to the repetitive frequencies of arginine, lysine and glycine (Table 3.6). In addition, the substitution of residue A395 to an acid residue (glutamic acid) was also repeatedly observed (Table 3.6). Furthermore, the residue G397 was also changed in favor to aliphatic and basic amino acids due to the occurrence of lysine, arginine, valine, asparagine, leucine. Interestingly, residue G397 of CYP106A2 mutants 5 and 6 was substituted by a Stop codon. Therefore, these mutant proteins were shorter than the wild type and other mutant CYP106A2 proteins by 14 amino acids. CYP106A2 mutant 44 did not change at residue G397, but residue T396 was substituted by proline. Combining the replacements at positions A395 and G397, mutant CYP106A2 A395E/G397Stop, CYP106A2 A395R/G397P, CYP106A2 A395K and CYP106A2 A395K/G397N occurred twice.

Table 3.6: Sequencing of the active substrate recognition site (SRS) 6 mutants. The changed nucleotide was colored in red.

Mutant Abbreviation	Mutant points	Nucleotides changed
Mutant 2	A395R/G397H	gcg → agg; ggt → cac
Mutant 3	A395H/G397L	gcg → cat; ggt → cta
Mutant 5	A395E/G397Stop	gcg → gag; ggt → tga
Mutant 6	A395E/G397Stop	gcg → gag; ggt → tga
Mutant 20	A395G/G397C	gcg → ggg; ggt → tgt
Mutant 21	A395R/G397K	gcg → agg; ggt → aaa
Mutant 23	A395G/G397V	gcg → ggg; ggt → gta
Mutant 24	A395N/G397R	gcg → aat; ggt → cgt
Mutant 26	A395S/G397K	gcg → tca; ggt → aaa
Mutant 28	A395R/G397V	gcg → agg; ggt → gta
Mutant 29	A395G/G397D	gcg → ggg; ggt → gat
Mutant 30	A395P/G397L	gcg → cca; ggt → cta
Mutant 33	A395R/G397R	gcg → agg; ggt → agg
Mutant 36	A395P/G397K	gcg → cca; ggt → aaa
Mutant 37	A395K/G397N	gcg → aaa; ggt → aat
Mutant 38	A395K	gcg → aag
Mutant 39	A395W/G397K	gcg → tgg; ggt → aaa
Mutant 41	A395R/G397P	gcg → cgt; ggt → cca
Mutant 43	A395K/G397N	gcg → aaa; ggt → aat
Mutant 44	A395V/T396P	gcg → gta; acc → ccc
Mutant 45	A395K	gcg → aag
Mutant 46	A395E/G397V	gcg → gag; ggt → gta
Mutant 47	A395R/G397P	gcg → cgg; ggt → cct
Mutant 48	A395Q/G397R	gcg → caa; ggt → cga

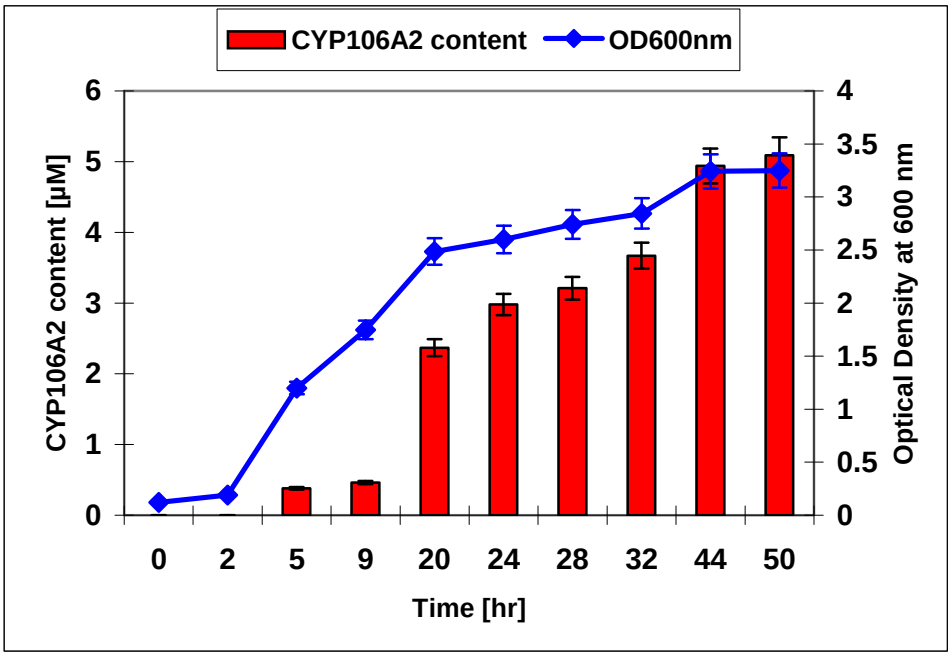
3.2. Improvement of catalytic efficiency towards 11α-hydroxyprogesterone

My work focused on the mutants which showed high 11α-hydroxylation activity. Mutant CYP106A2 A395W/G397K showed an interesting regio-selectivity to convert progesterone mainly in 11α-position. Furthermore, mutant CYP106A2 A395I, one of the most interesting mutants from Virus (Virus, 2006), was also able to produce a high amount of 11α-hydroxyprogesterone with 97.2% of the relative value compared with 15β-hydroxylated product. Unfortunately, both mutant enzymes had a 50% lower turnover rate than CYP106A2 wild type. Therefore, these 2 mutants were chosen for further mutation to improve the catalytic efficiency but still retain high regio-selective hydroxylation towards C-11.

3.2.1. Heterologous expression of mutants CYP106A2 A395I and CYP106A2 A395W/G397K

Since for detailed biochemical studies of the mutants, sufficient amounts of the protein are necessary, at first the expression and purification procedure for CYP106A2 mutants were optimized. Mutants CYP106A2 A395I and CYP106A2 A395W/G397K, the parental enzymes for further mutagenesis experiments, were chosen and the expression in *E.coli* JM109 was done as described in section 2.2.7. For this, 5 ml of samples were taken after 2, 5, 9, 20, 24, 28, 32, 44 and 50 hr to check the pH value, OD_{600} and cytochrome P450 content. Because the optical density deviation was linear in the range from 0 - 1.2, samples with an optical density of more than 1.2 were diluted 2 - 3 fold. The samples (1 ml) were centrifuged to remove the supernatants. The pellets were dissolved in potassium phosphate buffer pH 7.4 and subsequently broken down by sonification. After centrifugation, the supernatants were measured for CO-difference spectra. The results are represented in Figure 3.4 and 3.5.

A)



B)

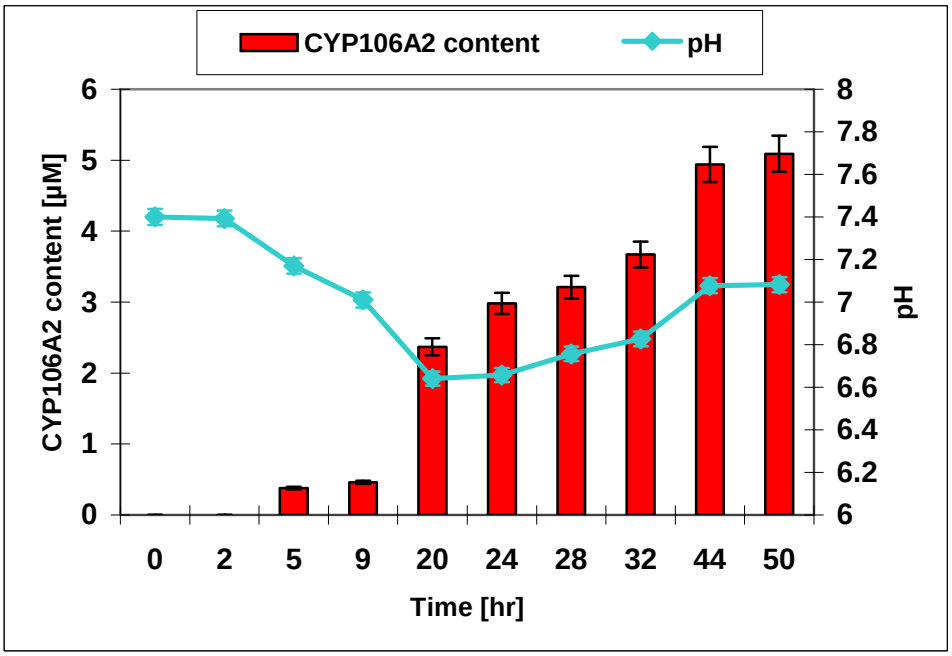
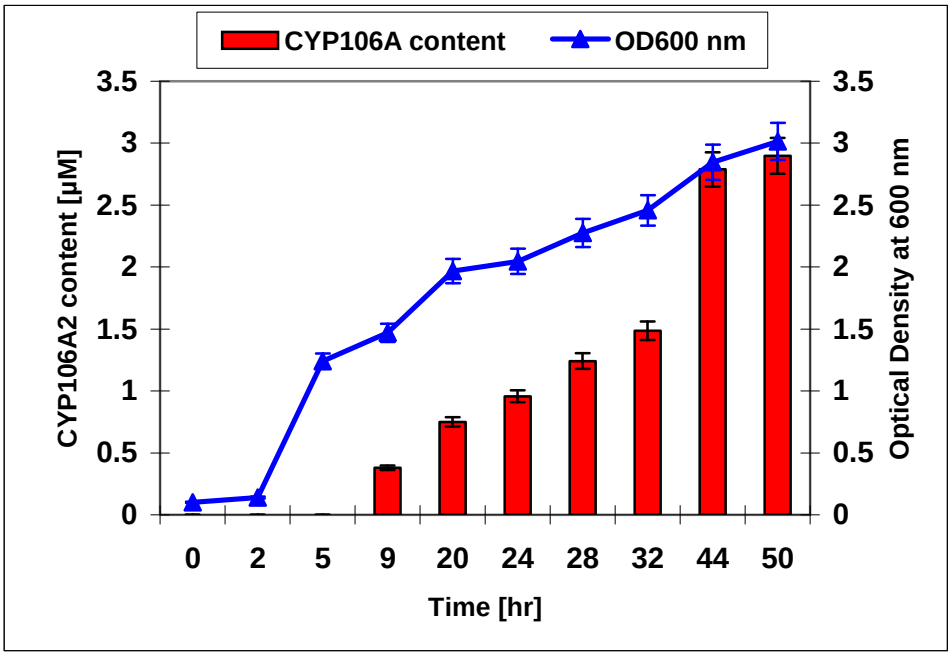


Figure 3.4: Heterologous expression of the CYP106A2 A395I mutant in a 2 L Erlenmayer flask containing 1 L TB media at 30°C for 50 hr. The data shown here represent 3 independent experiments. Graphic representation of (A) the progress of cell density (blue line) and CYP106A2 content (red bars) and (B) the progress of pH change (aquamarine line) and CYP106A2 content (red bars).

A)



B)

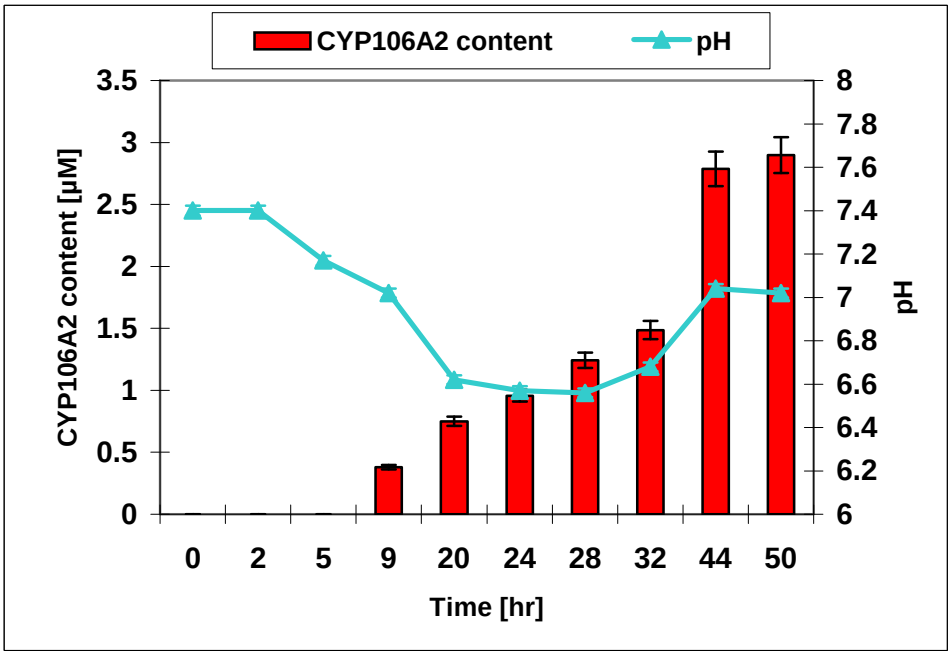


Figure 3.5: Heterologous expression of the CYP106A2 A395W/G397K mutant in a 2 L Erlenmayer flask containing 1 L TB media at 30°C for 50 hr. The data shown here represent 3 time independent experiments. Graphic representation of (A) the progress of cell density (blue line) and CYP106A2 content (red bars) and (B) the progress of pH change (aquamarine line) and CYP106A2 content (red bars).

Figures 3.4 and 3.5 showed the relationship between the cell density, pH value and the expression of cytochrome P450 during the time interval for 2 of the selected mutants (CYP106A2 A395I and CYP106A2 A395W/G397K). The cell density correlated with the concentration of CYP106A2, where the higher cell density corresponded to more CYP106A2 content. The pH value at the beginning of expression was fixed at 7.4, but during the expression the pH dropped down after 2 hr and reached 6.6 ± 0.1 (at 20 - 24 hr) and then increased again to 7.0 after 44 hr where CYP106A2 was produced with the highest amount. The cell density and pH changes were not different between the 2 mutants (CYP106A2 A395I and CYP106A2 A395W/G397K). However, their P450 concentrations gained maxima at 5.09 µM and 2.9 µM after 50hr, respectively.

3.2.2. Site directed mutagenesis of CYP106A2 A395I and CYP106A2 A395W/G397K

In order to improve the catalytic efficiency of the 2 parental mutants (CYP106A2 A395I and CYP106A2 A395W/G397K), they were further mutated at positions which were reported in the literature as leading to increased catalytic activities. According to the work of Virus (Virus, 2006) and Rauschenbach (Rauschenbach, 1993), the mutations D217V, A243V, A106T, A106T/R409L, T89N/A106T/R409L, T89N/A106T/Q189K/R409L, F165L showed higher turnover rates towards progesterone conversion (1.18-, 1.05-, 1.43-, 1.43-, 1.44-, 1.13- and 2.61-fold, respectively, compared with CYP106A2 wild type).

Mutants CYP106A2 A106T, CYP106A2 D217V, CYP106A2 A243V and CYP106A2 A106T/R409L had been identified through the screening from the first generation mutagenesis library of CYP106A2 using error prone PCR. The double mutant CYP106A2 A106T/R409L showed the highest expression yield as well as the fastest catalytic activity. Therefore, this mutant was chosen as a template to carry out the second generation mutagenesis library. Mutants CYP106A2 T89N/A106T/R409L and CYP106A2 T89N/A106T/Q189K/R409L were obtained from this second round mutagenesis (Virus, 2006). Moreover, to evaluate the effect of each mutation not only on enzyme activity but also on selectivity of hydroxylation, mutations T89N and R409L were separately studied. For mutant CYP106A2 F165L, a 2.5-fold higher progesterone conversion rate was reported (Rauschenbach, 1993).

Finally, a highly conserved threonine (T) within the I-helix of cytochrome P450 family is thought to be involved in the proton shuttle and is important for proton delivery and molecular oxygen activation. The corresponding residue in CYP106A2 is threonine at

position 247 (T247), which is placed in the active site near to heme (Lisurek *et al.*, 2008). Interestingly, next to this residue, another threonine, T248, is found. With a desire to improve catalytic efficiency of 2 parental mutants (CYP106A2 A395I, CYP106A2 A395W/G397K) and to explore the role of residues T247 and T248 for the properties of CYP106A2, the following mutations were proceduced: T247A, T247V, T247S, T247P, T247W, T248A and T248V, respectively.

Site-specific mutagenesis in this study was performed using the Strategene Quik Change Mutagenesis Kit as described in section 2.2.5. The positive clones, after analysis by restriction digestion, were sequenced (MWG Company, Ebersberg, Germany) to confirm the mutation.

3.2.3. Quick screening of the "site directed mutagenesis"CYP106A2 mutant library in micro titer plates

To screen each of the 28 CYP106A2 mutants individually as described in section 2.2.7, it would take a long time with high costs. Therefore, an efficient screening system which was established by Virus (Virus, 2006) was applied to screen the "new mutant" collection faster. The principle of this method was derived from a published procedure (Apple *et al*., 2005). The authors determined the conversion rate of 11-deoxycortisol to cortisol of a CYP11B1 expressing *Schizosaccharomyces pombe* culture by fluorescence measurements. Hannemann *et al.* (Hannemann *et al*, 2006) modified this method to determine the activity of the steroid hydroxylating CYP106A2 in a micro titer plate format. The vectors pACYC_FHH2 containing the CYP106A2 mutants and pBAR_Twin containing Adx (4 - 108) and AdR were coexpressed in *E.coli* cells in micro titer plates as described in section 2.2.13. The hydroxylation reaction was followed measuring the fluorescence of the mixture with an excitation wavelength of 489 nm and an emission wavelength of 535 nm. The measurement results are shown in Table 3.7.

Mutants with a relative fluorescence unit (R.F.U) of more than 10000 for the 3 replicates were chosen for the next kinetic assay. Table 3.7 showed that 13 mutants were similar as CYP106A2 wild type. The 2 parental mutants (CYP106A2 A395I and CYP106A2 A395W/G397K) had R.F.U values of 7244 and 9521, respectively which was a half R.F.U value of CYP106A2 wild type. Mutants CYP106A2 A106T/A395I and CYP106A2 A106T/A395I/R409L revealed the highest R.F.U value (23878 ± 4264 and 24432 ± 2332, respectively).

Some substitutions such as A106T, A243V, A106T/R409L seemed to affect the activity due to increasing the R.F.U value of the new mutants. On the other hand, substitution R409L alone was not able to bring the improvement of catalytic activity to the new mutants. Similarly, 11 out of 14 mutants at the residues T247 and T248 showed a smaller R.F.U value compared with parental mutants. Only 3 mutants (CYP106A2 T247V/A395I, CYP106A2 T247W/A395W/G397K and CYP106A2 T248V/A395I) showed an increased activity due to a higher R.F.U value compared with the parental mutants.

Table 3.7: Representation of mutant positions in the micro titer plate (upper part) and data sheet of fluorescence (R.F.U) measurements (lower part). Each mutant was checked 3 times except for CYP106A2 A106T/A395I/R409L, CYP106A2 A106T/A395W/G397K/R409L and CYP106A2 T89N/A106T/A395I/R409L (repeated twice). Mutants CYP106A2 A395I, CYP106A2 A395W/G397K and CYP106A2 wild type were used as controls from position 4H to 12H (filled in black). The selected clones for further studies from this screening are filled in grey.

Position	1	2	3	4	5	6	7	8	9	10	11	12
A	T89N/A395I	T89N/A395I	T89N/A395I	T89N/39	T89N/39	T89N/39	A106T/A395I	A106T/A395I	A106T/A395I	A106T/39	A106T/39	A106T/39
B	F165L/A395I	F165L/A395I	F165L/A395I	F165L/39	F165L/39	F165L/39	D217V/A395I	D217V/A395I	D217V/A395I	D217V/39	D217V/39	D217V/39
C	A243V/A395I	A243V/A395I	A243V/A395I	A243V/39	A243V/39	A243V/39	A395I/R409L	A395I/R409L	A395I/R409L	39/R409L	39/R409L	39/R409L
D	T247A/A395I	T247A/A395I	T247A/A395I	T247A/39	T247A/39	T247A/39	T247V/A395I	T247V/A395I	T247V/A395I	T247V/39	T247V/39	T247V/39
E	T247F/A395I	T247F/A395I	T247F/A395I	T247F/39	T247F/39	T247F/39	T247S/A395I	T247S/A395I	T247S/A395I	T247S/39	T247S/39	T247S/39
F	T247W/A395I	T247W/A395I	T247W/A395I	T247W/39	T247W/39	T247W/39	T248A/A395I	T248A/A395I	T248A/A395I	T248A/39	T248A/39	T248A/39
G	T248V/A395I	T248V/A395I	T248V/A395I	T248V/39	T248V/39	T248V/39	2 mutants/A395I	2 mutants/A395I	2 mutants/39	2 mutants/39	3 mutants/A395I	3 mutants/A395I
H	3 mutants/39	3 mutants/39	3 mutants/39	A395I	A395I	A395I	39	39	39	WT	WT	WT

R.F.U	1	2	3	4	5	6	7	8	9	10	11	12
A	10431	13708	12025	5488	5003	4703	21413	20846	29375	17089	18642	10731
B	10876	11033	7534	3477	3905	1076	15460	12287	10254	3051	3682	4653
C	12248	10713	10068	19311	16749	17913	8456	8607	8903	4651	6830	7796
D	6046	7064	4068	6342	3091	3002	9387	11006	10387	6312	8689	7706
E	3781	2542	1096	8843	10346	8422	3311	2009	5791	4403	6123	7197
F	1338	1503	714	10653	12763	18897	8245	7467	5078	8321	6012	6392
G	11375	11937	9011	9407	8450	7377	24033	26964	22300	18564	13120	18256
H	6701	4319	4888	8012	6732	6989	8901	9903	9760	16897	15409	13487

39: CYP106A2 A395W/G397K
3 mutants/39 : CYP106A2 T89N/A106T/A395W/G397K/R409L
3 mutants/39 : CYP106A2 T89N/A106T/A395W/G397K/R409L
2 mutants/A395I: CYP106A2 A106T/A395I/R409L
2 mutants/39: CYP106A2 A106T/A395W/G397K/R409L
R.F.U: relative fluorescence unit

3.2.4. Kinetic investigations of the selected mutants

Plasmids pACYC_FHH2 containing selected CYP106A2 mutants and wild type were transformed and heterologously expressed in *E.coli* JM109 cells as described in section 2.2.7. The cells from the expressed cultures were harvested by centrifugation at 6000 rpm for 10 min and stored at –20 °C until use.

3.2.4.1 Purification and spectrophotometric characterization of CYP106A2

To investigate the kinetics of the newly proceduced mutants, the 16 recombinant mutant and wild type proteins were purified as described in section 2.2.8. The purification steps and purified CYP106A2 are shown in SDS-PAGE (Figure 3.6). Cytochrome P450 concentration was found to be decreased in every purification step and is shown in Table 3.8.

The highest yield of CYP106A2 expression was observed in mutants CYP106A2 A243V/A395W/G397K (1440 nmol/L culture), CYP106A2 A243V/A395W/G397K (1457 nmol/L culture) and CYP106A2 A395I (1550 nmol/L culture). However, the CYP106A2 content was reduced by approximately 32%, 18% and 12% after applying on the DEAE Sepharose column, the HAP column and the gel filtration column, respectively.

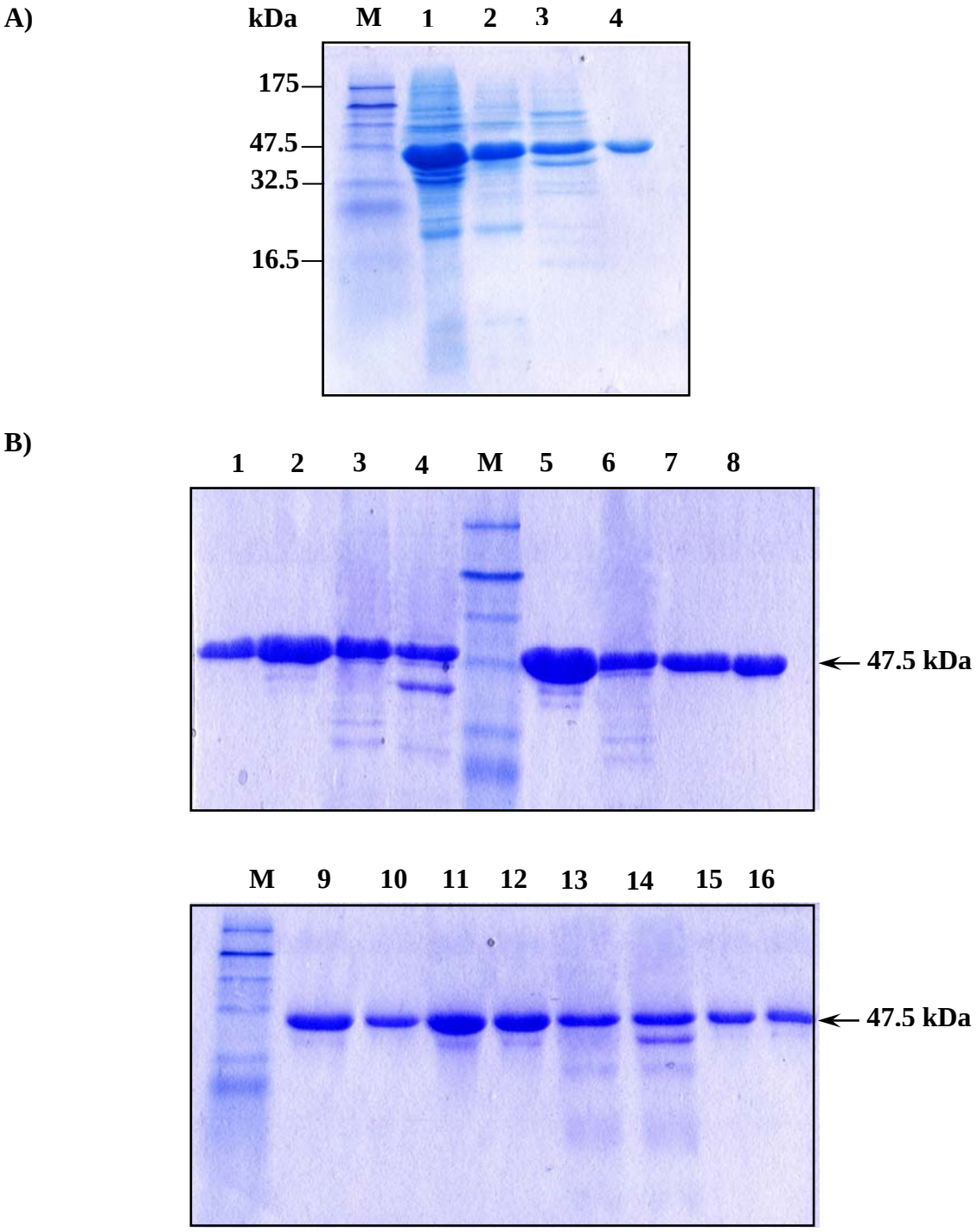


Figure 3.6: SDS-PAGE of the purification procedure and purified enzymes of the mutants and wild type. **A)** The purification procedure of CYP106A2 wild type (as an example). **M**: marker; **1**: supernatant; **2**: after DEAE-Sepharose column; **3**: after HAP column; **4**: after Superdex 75 column. **B)** The purified proteins of mutants. **M**: marker; **1**: CYP106A2 T89N/A395I; **2**: CYP106A2 A106T/A395I; **3**: CYP106A2 A106T/A395W/G397K; **4**: CYP106A2 A243V/A395I; **5**: CYP106A2 A243V/A395W/G397K; **6**: CYP106A2 D217V/A395I; **7**: CYP106A2 F165L/A395I; **8**: CYP106A2 T247V/A395I; **9**: CYP106A2 T247W/A395W/G397K; **10**: CYP106A2 T248V/A395I; **11**: CYP106A2 A106T/A395I/R409L; **12**: CYP106A2 A106T/A395W/G397K/R409L; **13**: CYP106A2 T89N/A106T/A395I/R409L; **14**: CYP106A2 A395I; **15**: CYP106A2 A395W/G397K; **16**: CYP106A2 wild type.

Table 3.8: The yield of cytochrome P450 after different purification step.

Mutant	Crude enzyme solution [nmol/L]	After DEAE column [nmol/L]	After HAP column [nmol/L]	After Superdex 75 column [nmol/L]	CYP106A2/ total protein [nmol/mg]
T89N/A395I	845	622	517	483	7.94
A106T/A395I	1678	1320	1004	846	12.05
A106T/A395W/ G397K	804	576	320	273	5.97
A243V/A395I	1457	1201	974	878	10.54
A243V/A395W/ G397K	1440	867	532	448	7.21
D217V/A395I	1245	955	675	467	7.84
F165L/A395I	1062	631	403	326	6.72
T247V/A395I	759	532	400	339	6.06
T247W/A395W/ G397K	879	642	437	372	6.93
T248V/A395I	823	600	401	359	6.80
A106T/A395I/ R409L	702	458	306	242	5.26
A106T/A395W/ G397K/R409L	520	350	201	183	4.65
T89N/A106T/ A395I/R409L	847	513	420	361	6.72
A395I	1550	1002	780	692	10.76
A395W/G397K	877	514	382	311	6.67
Wild type	1273	780	662	567	8.27

Purified CYP106A2 mutants and wild type were measured using UV-Vis and CO-difference spectra as described in section 2.2.9 (Figure 3.7). The concentration of enzymes was 5 μM in 50 mM potassium phosphate buffer (pH 7.4) for UV-Vis and 10 μM for CO-different spectra measurements, respectively.

All protein samples were homogenous and showed the typical oxidized low-spin state of the cytochrome P450 with maximum absorption at 568 nm and 534 nm (Q Bands), the Soret band at 417 nm, the UV band at 358 nm which was caused by electron transitions of the heme chromophore, as well as the protein band at 280 nm, that was evoked by the

absorption of the aromatic amino acids. Moreover, the mutants and CYP106A2 wild type exhibited the characteristics of reduced P450, which was bound by carbon monoxide, to yield the classic CO-difference spectra with a maximum at approximately 450 nm (451.4 nm except for CYP106A2 A243V/A395W/G397K, CYP106A2 T247V/A395W/G397K and CYP106A2 T89N/A106T/A395I/R409L, which showed the Soret peaks at 450.9 nm). Purification seemed to decrease the formation of cytochrome P420. Only 5 of the purified mutant proteins (CYP106A2 T89N/A106T, CYP106A2 A243V/A395W/G397K, CYP106A2 T247V/A395W/G397K, CYP106A2 T89N/A106T/A395I/R409L and CYP106A2 A395W/G397K) showed the inactive P450 forms.

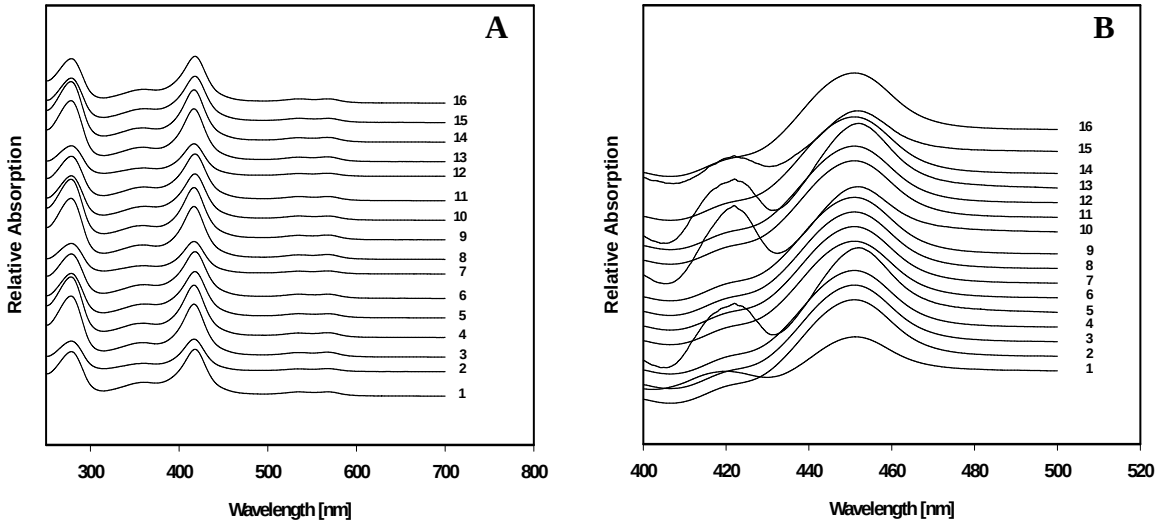


Figure 3.7: UV-Vis (A) and CO-different (B) spectra of the CYP106A2 wild type and mutants. The concentration of the enzymes was 5 μM in 10 mM potassium phosphate buffer (pH 7.4) for UV-Vis and 10 μM for CO-different spectra measurements, respectively. **1**: CYP106A2 T89N/A395I; **2**: CYP106A2 A106T/A395I; **3**: CYP106A2 A106T/A395W/G397K; **4**: CYP106A2 A243V/A395I; **5**: CYP106A2 F165L/A395I; **6**: CYP106A2 A243V/A395W/G397K; **7**: CYP106A2 D217V/A395I; **8**: CYP106A2 T247V/A395I; **9**: CYP106A2 T248V/A395I; **10**: CYP106A2 T247W/A395W/G397K; **11**: CYP106A2 A106T/A395I/R409L; **12**: CYP106A2 A106T/A395W/G397K/R409L; **13:** CYP106A2 T89N/A106T/A395I/R409L; **14:** CYP106A2 A395I; **15**: CYP106A2 A395W/G397K; **16**: CYP106A2 wild type.

3.2.4.2 Kinetic analysis of CYP106A2 mutants concerning progesterone conversion

The activity of the purified proteins was reconsituted *in vitro* using truncated bovine adrenodoxin (Adx 4 - 108) and adrenodoxin reductase (AdR). The system contained 0.25 µM of CYP106A2, 0.5 µM of AdR and 5 µM of Adx. Progesterone concentrations ranged from 17.5 - 525 µM. The reaction was carried out in a thermal shaker at 30°C for 10 min. The 20β-hydroxyprogesterone was used as internal standard (3 µl of a 10 mM solution). The steroids subsequently were analyzed using HPLC and the amount of products was calculated using the equation (f(x) = ax/(b+x)); standard deviations were derived from 3 independent experiments. The results are shown in Figure 3.8a, 3.8b, 3.8c and Table 3.9 and 3.10.

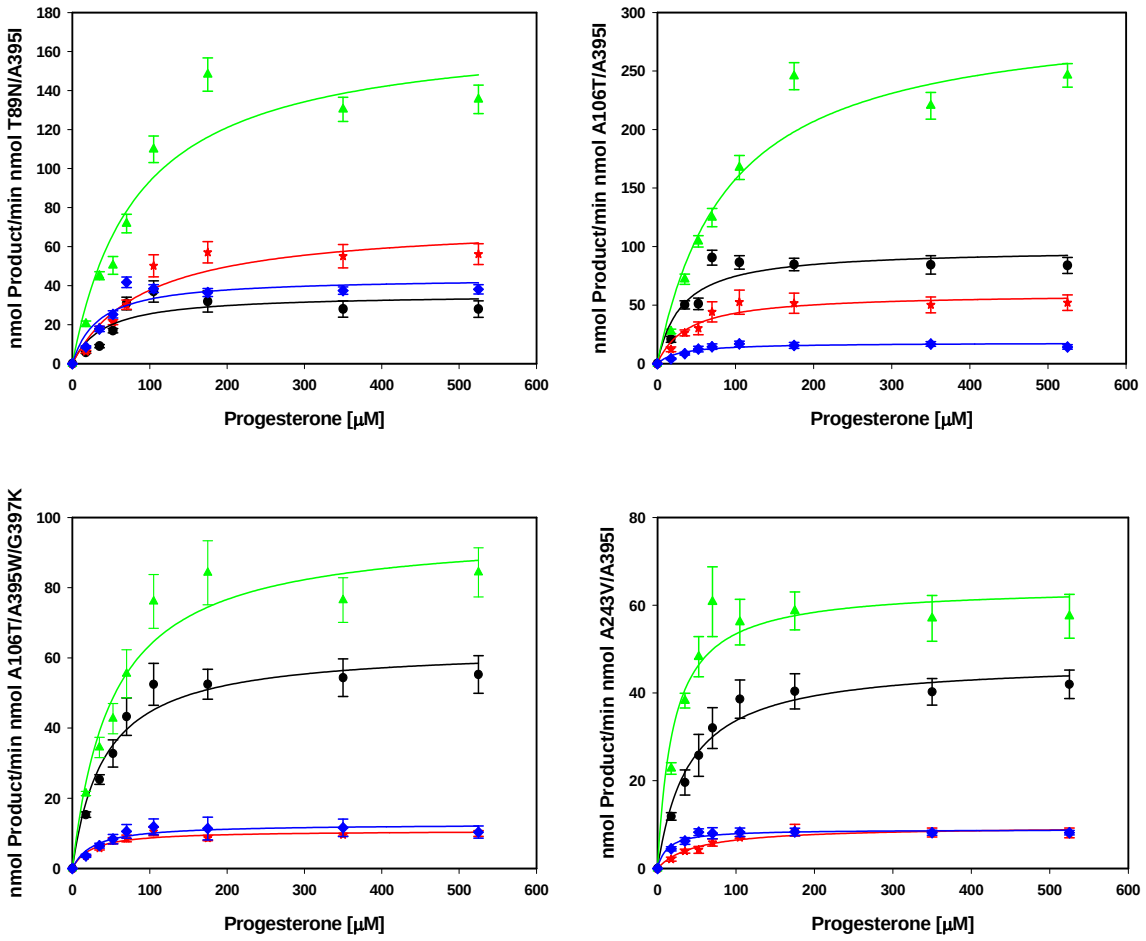


Figure 3. 8a: Representation of the progesterone conversion rates depending on substrate concentration of the mutants CYP106A2 T89N/A395I, CYP106A2 A106T/A395I, CYP106A2 A106T/A395W/G397K, CYP106A2 A243V/A395I. Black lines and circles: 15β-hydroxyprogesterone; green lines and triangles: 11α-hydroxyprogesterone; red lines and stars: 9α-hydroxyprogesterone; blue lines and diamonds: 6β-hydroxyprogesterone.

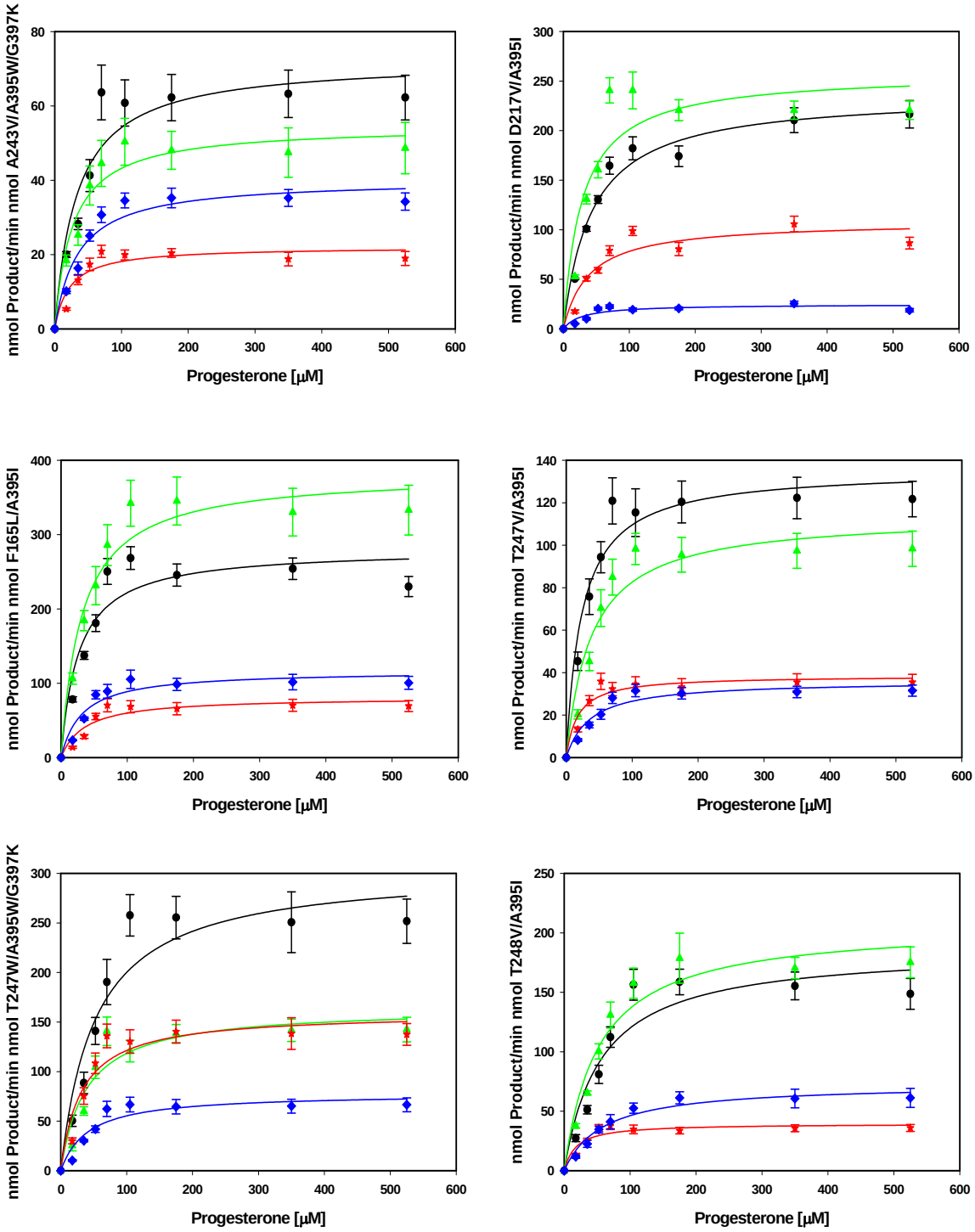


Figure 3. 8b: Representation of the progesterone conversion rates depending on substrate concentration of the mutants CYP106A2 A243V/A395W/G397K, CYP106A2 D217V/A395I, CYP106A2 F165L/A395I, CYP106A2 T247V/A395I, CYP106A2 T247W/A395W/G397K and CYP106A2 T248V/A395I. Black lines and circles: 15β-hydroxyprogesterone; green lines and triangles: 11α-hydroxyprogesterone; red lines and stars: 9α-hydroxyprogesterone; blue lines and diamonds: 6β-hydroxyprogesterone.

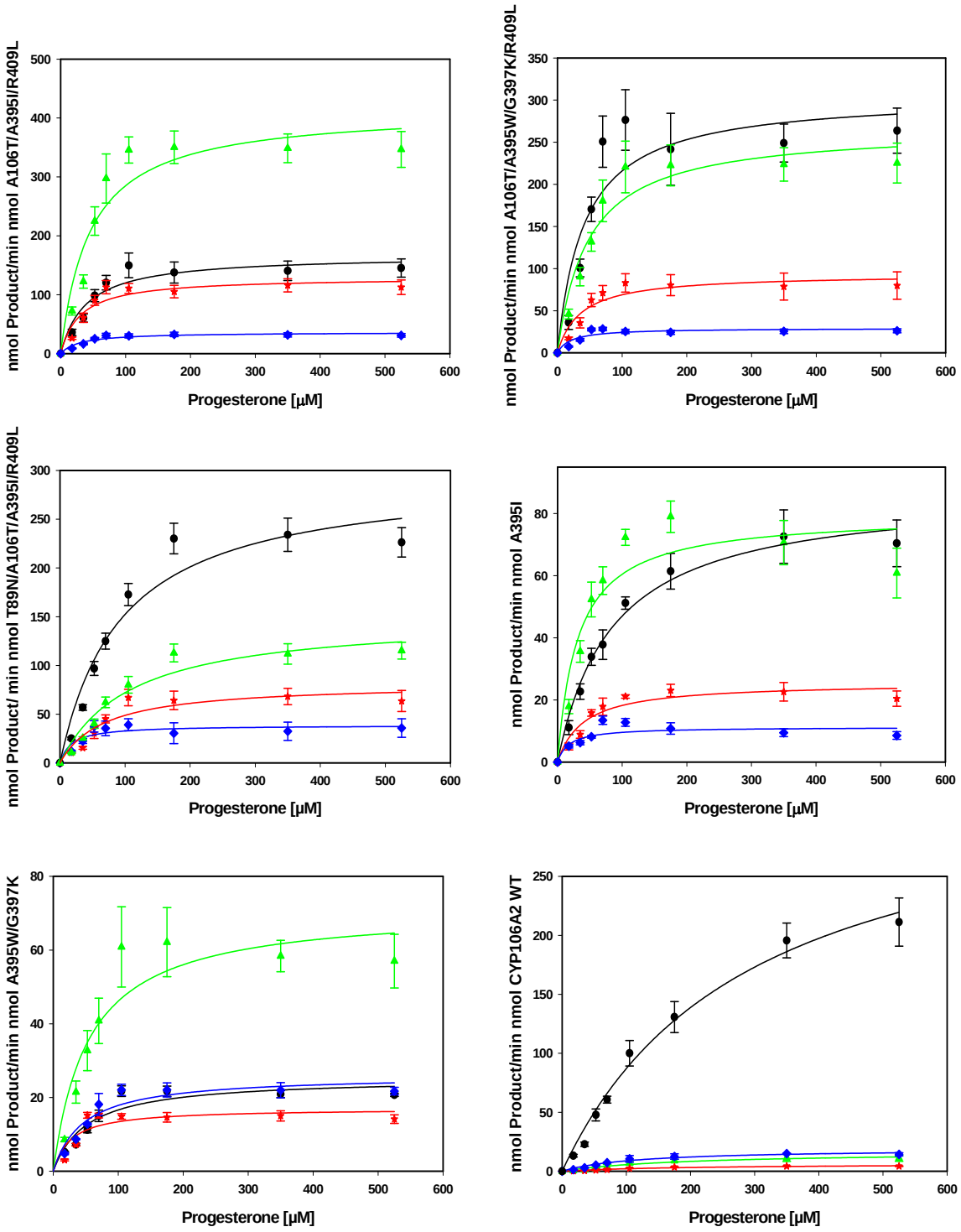


Figure 3. 8c: Representation of the progesterone conversion rates depending on substrate concentration of the mutants CYP106A2 A106T/A395I/R409L, CYP106A2 A106T/A395W/G397K/R409L, CYP106A2 T89N/A106T/A395I/R409L, CYP106A2 A395I, CYP106A2 A395W/G397K and the CYP106A2 wild type. Black lines and circles: 15β-hydroxyprogesterone; green lines and triangles: 11α-hydroxyprogesterone; red lines and stars: 9α-hydroxyprogesterone; blue lines and diamonds: 6β-hydroxyprogesterone.

Table 3.9: Apparent K_m and V_{max} values of the CYP106A2 mutants resulting from calculations. The data presented in Figure 3. 8a, b, c (*part 1*).

	K_m [µM]	V_{max} [nmol/min nmol P450]	kcat/K_m [M^{-1} sec^{-1}] x 10^3
T89N/A395I			
15β-OH-P	40.0 ± 24.9	35.7 ± 6.1	14.9 ± 17.7
11α-OH-P	83.7 ± 26.7	171.7 ± 20.0	34.2 ± 13.6
9α-OH-P	93.7 ± 34.9	72.9 ± 9.9	13.0 ± 6.4
6β-OH-P	33.7 ± 14.8	44.1 ± 5.0	21.8 ± 13.2
A106T/A395I			
15β-OH-P	31.2 ± 12.7	97.9 ± 10.0	52.3 ± 28.1
11α-OH-P	91.0 ± 23.6	300.9 ± 28.2	55.1 ± 16.8
9α-OH-P	39.2 ± 11.4	60.0 ± 4.8	25.5 ± 8.8
6β-OH-P	29.1 ± 10.5	17.9 ± 1.6	10.3 ± 4.7
A106T/A395W/G397K			
15β-OH-P	41.6 ± 8.5	63.1 ± 3.6	25.3 ± 5.7
11α-OH-P	51.6 ± 12.4	96.4 ± 7.0	31.1 ± 8.6
9α-OH-P	24.4 ± 6.9	10.8 ± 0.7	7.4 ± 2.5
6β-OH-P	26.7 ± 8.6	12.7 ± 1.0	7.9 ± 3.1
A243V/A395I			
15β-OH-P	40.4 ± 7.2	47.3 ± 2.3	19.5 ± 3.8
11α-OH-P	20.2 ± 6.1	64.3 ± 4.0	53.1 ± 23.6
9α-OH-P	40.1 ± 10.8	9.6 ± 0.6	4.0 ± 1.3
6β-OH-P	12.5 ± 3.4	8.9 ± 0.4	11.9 ± 3.7
A243V/A395W/G397K			
15β-OH-P	33.4 ± 11.3	72.3 ± 6.3	36.1 ± 15.0
11α-OH-P	26.4 ± 7.2	54.6 ± 3.5	34.5 ± 10.8
9α-OH-P	21.9 ± 9.2	22.1 ± 2.0	16.8 ± 9.4
6β-OH-P	34.5 ± 9.1	40.2 ± 2.8	19.4 ± 5.9
D217V/A395I			
15β-OH-P	43.3 ± 7.4	236.8 ± 11.5	91.1 ± 16.9
11α-OH-P	28.4 ± 12.0	258.1 ± 26.3	151.5 ± 85.9
9α-OH-P	38.7 ± 14.2	108.3 ± 10.8	46.6 ± 21.8
6β-OH-P	28.7 ± 14.5	24.8 ± 3.0	14.4 ± 11.0

Table 3.9: Apparent K_m and V_{max} values of the CYP106A2 mutants resulting from calculations. The data presented in Figure 3. 8a, b, c (*part 2*).

	K_m [µM]	V_{max} [nmol/min nmol P450]	kcat/K_m [M^{-1} sec^{-1}] x 10^3
F165L/A395I			
15β-OH-P	26.6 ± 10.0	280.7 ± 24.8	175.9 ± 83.8
11α-OH-P	31.6 ± 7.6	382.7 ± 23.3	201.8 ± 54.7
9α-OH-P	35.3 ± 14.5	81.1 ± 8.7	38.3 ± 21.0
6β-OH-P	31.5 ± 10.4	116.7 ± 9.7	61.7 ± 24.8
T247V/A395I			
15β-OH-P	23.8 ± 5.7	135.6 ± 7.3	95.0 ± 18.8
11α-OH-P	38.1 ± 11.3	114.1 ± 9.1	49.9 ± 13.9
9α-OH-P	17.1 ± 6.0	38.7 ± 2.6	37.7 ± 16.1
6β-OH-P	36.1 ± 10.0	36.1 ± 2.7	16.7 ± 5.4
T247W/A395W/G397K			
15β-OH-P	52.2 ± 16.7	304.5 ± 29.6	97.2 ± 38.1
11α-OH-P	38.8 ± 14.2	164.3 ± 16.4	70.6 ± 32.8
9α-OH-P	31.4 ± 10.4	159.4 ± 13.2	84.6 ± 34.1
6β-OH-P	40.9 ± 14.8	78.0 ± 7.9	31.8 ± 14.6
T248V/A395I			
15β-OH-P	55.6 ± 19.3	186.6 ± 20.1	55.9 ± 24.5
11α-OH-P	51.7 ± 12.5	207.1 ± 15.2	66.8 ± 18.4
9α-OH-P	17.8 ± 7.1	39.6 ± 3.1	37.1 ± 19.0
6β-OH-P	58.0 ± 11.9	73.1 ± 4.7	21.0 ± 4.8
A106T/A395I/R409L			
15β-OH-P	39.1 ± 11.9	166.9 ± 13.8	71.1 ± 25.8
11α-OH-P	45.7 ± 14.9	415.2 ± 39.2	151.4 ± 60.5
9α-OH-P	30.4 ± 10.8	129.5 ± 11.1	70.0 ± 31.4
6β-OH-P	29.7 ± 9.3	36.2 ± 2.8	20.3 ± 7.6
A106T/A395W/G397K/ R409L			
15β-OH-P	41.7 ± 18.2	305.8 ± 37.8	122.2 ± 73.8
11α-OH-P	47.6 ± 13.2	266.7 ± 21.8	93.4 ± 30.4
9α-OH-P	35.7 ± 12.4	93.4 ± 8.6	43.6 ± 18.9
6β-OH-P	20.9 ± 10.8	29.2 ± 3.2	23.3 ± 18.3

Table 3.9: Apparent K_m and V_{max} values of the CYP106A2 mutants resulting from calculations. The data presented in Figure 3. 8a, b, c (*part 3*).

	K_m [µM]	**V_{max} [nmol/min nmol P450]**	**kcat/K_m [M^{-1} sec^{-1}] x 10^3**
T89N/A106T/A395I/ R409L			
15β-OH-P	94.1 ± 25.0	295.7 ± 28.8	52.4 ± 16.5
11α-OH-P	107.8 ± 29.6	149.8 ± 15.7	23.2 ± 7.6
9α-OH-P	67.7 ± 26.3	81.8 ± 10.5	20.1 ± 10.4
6β-OH-P	17.7 ± 9.5	38.6 ± 4.0	36.3 ± 30.3
A395I			
15β-OH-P	85.5 ± 10.7	87.2 ± 3.9	17.0 ± 2.3
11α-OH-P	31.6 ± 12.2	79.6 ± 7.8	42.0 ± 20.9
9α-OH-P	40.1 ± 12.6	25.5 ± 2.2	10.6 ± 4.0
6β-OH-P	17.8 ± 14.7	11.2 ± 1.8	10.5 ± 3.6
A395W/G397K			
15β-OH-P	68.6 ± 29.3	26.4 ± 3.7	6.4 ± 3.9
11α-OH-P	54.7 ± 19.8	71.3 ± 8.0	21.7 ± 10.1
9α-OH-P	26.8 ± 13.7	17.0 ± 2.0	10.6 ± 8.2
6β-OH-P	46.2 ± 14.1	26.1 ± 2.3	9.4 ± 3.5
CYP106A2 WT			
15β-OH-P	292.9 ± 48.7	342.0 ± 29.1	19.5 ± 3.7
11α-OH-P	203.4 ± 66.9	16.8 ± 2.5	1.4 ± 0.6
9α-OH-P	209.5 ± 66.4	6.4 ± 0.9	0.5 ± 0.3
6β-OH-P	117.2 ± 27.3	19.1 ± 1.7	2.7 ± 0.8

Table 3.10: Regio-specificity of CYP106A2 mutants towards progesterone conversion.

	15β-OH-P [%]	**11α-OH-P [%]**	**9α-OH-P [%]**	**6β-OH-P [%]**
T89N/A395I	100	426.2 ± 7.6	176.3 ± 4.8	125.8 ± 6.1
A106T/A395I	100	274.3 ± 6.9	59.8 ± 2.6	18.4 ± 0.5
A106T/A395W/ G397K	100	148.5 ± 7.0	18.0 ± 0.3	21.1 ± 0.7
A243V/A395I	100	144.5 ± 5.4	20.3 ± 0.7	20.5 ± 0.8
A243V/A395W/ G397K	100	77.1 ± 2.8	31.6 ± 1.8	55.4 ± 3.8
D217V/A395I	100	114.0 ± 8.2	46.4 ± 2.6	10.9 ± 0.4
F165L/A395I	100	134.3 ± 9.3	28.1 ± 0.6	41.0 ± 1.8
T247V/A395I	100	80.6 ± 5.8	29.2 ± 0.2	25.7 ± 0.8
T247W/A395W/ G397K	100	56.1 ± 4.8	55.6 ± 7.1	26.5 ± 1.7
T248V/A395I	100	112.2 ± 3.3	23.7 ± 0.9	38.9 ± 1.8
A106T/A395I/ R409L	100	243.9 ± 9.8	79.6 ± 5.3	22.3 ± 0.3
A106T/A395W/ G397K/R409L	100	85.7 ± 5.1	31.1 ±1.1	10.2 ± 0.2
T89N/A106T/ A395I/R409L	100	49.0 ± 4.1	29.6 ± 0.4	16.2 ± 1.9
A395I	100	97.2 ± 2.1	32.1 ± 0.4	13.5 ± 0.1
A395W/G397K	100	280.5 ± 4.8	72.6 ± 0.8	105.2 ± 0.9
CYP106A2-WT	100	5.0 ± 0.8	1.5 ± 0.3	5.1 ± 0.8

As shown in Table 3.9, new CYP106A2 mutants with significantly improved catalytic efficiency along with a high percentage of 11α-hydroxyprogesterone formation were obtained. Three mutants (CYP106A2 D217V/A395I, CYP106A2 F165L/A395I and CYP106A2 A106T/A395I/R409L) exhibited a significantly increased apparent k_{cat}/K_m of 11α-hydroxyprogesterone formation (7.8-, 10.3- and 7.8-fold, respectively) compared with that of 15β-hydroxyprogesterone from CYP106A2 wild type. Moreover, these mutants also showed a higher relative amount of 11α-hydroxylated products being 114%, 134.3% and 243,9%, higher than that of the parental mutant CYP106A2 A395I (97.2%) (Table 3.10). Mutants CYP106A2 A106T/A395W/G397K, CYP106A2 A243V/A395I

and CYP106A2 T248V/A395I only improved the 11α-hydroxylation by 1.6-, 2.7- and 3.4-fold compared with 15β-hydroxylation of CYP106A2 wild type. However, these mutants produced more 11α-hydroxyprogesterone (148.5%, 144.5% and 112.2%, respectively) than 15β-hydroxyprogesterone. The new mutations affected not only in the velocity but also in the regio-specificity of enzymes. Despite increasing the catalytic activity, mutants CYP106A2 A243V/A395W/G397K, CYP106A2 T247V/A395I, CYP106A2 T247W/A395W/G397K, CYP106A2 A106T/A395W/G397K and CYP106A2 T89N/A106T/A395I/R409L showed a decrease of the 11α-hydroxyprogesterone formation compared with the parental enzyme (Table 3.10).

Interestingly, 2 mutants CYP106A2 T89N/A395I and CYP106A2 A106T/A395I clearly produced more 11α-hydroxyprogesterone (426.2% and 274.3%) as compared with the 15β-hydroxyprogesterone. The 11α-hydroxyprogesterone apparent k_{cat}/K_m showed little increase (1.8- and 2.8-fold) compared with that of 15β-hydroxyprogesterone from CYP106A2 wild type. Furthermore, the 11α-hydroxyprogesterone apparent k_{cat}/K_m of CYP106A2 T89N/A395I (34.2 ± 13.6 [$M^{-1}sec^{-1}x10^3$]) was less than that of the parental mutant CYP106A2 A395I (42.0 ± 20.9 [$M^{-1}sec^{-1}x10^3$]). However, the regio-selectivity towards 11α-hydroxyprogesterone was 4.4- and 2.8-fold, respectively, higher than that of the parental mutant CYP106A2 A395I. On the other hand, the 9α- and 6β-hydroxyprogesterone formations of these 2 mutants were also changed. Mutant CYP106A2 T89N/A395I showed a formation of 6β- and 9α-hydroxyprogesterone of 125.8%, 176.3%, respectively, compared to 15β-hydroxylation. In comparison with mutant CYP106A2 A395I, mutant CYP106A2 T89N/A395I produced 5.5-fold more 9α-hydroxyprogesterone and 9.3-fold more 6β-hydroxyprogesterone. Likewise, in the CYP106A2 A106T/A395I mutant, the relative values of 9α- and 6β-hydroxyprogesterone were 59.8% and 18.4%, demonstrating an 1.9- and 1.4-fold increase compared with CYP106A2 A395I mutant.

3.3. Progesterone conversion in whole-cell system

Steroid intermediate productions by whole-cell systems nowadays become more popular than using isolated enzymes due to time and costs (Straathof *et al*., 2002). To reveal the capacity of the CYP106A2 mutants in *in vivo* conversions, 7 out of 15 mutants were selected and investigated. The selected mutants were chosen according to a high catalytic

efficiency as well as 11α-hydroxyprogesterone formation. Coexpression of CYP106A2 mutants as well as the redox partner (Adx and AdR) was performed in JM109 *E.coli* cells.

3.3.1 Retention time of hydroxylated products in the HPLC

The whole-cell biotransformations of progesterone with CYP106A2 mutants were analyzed using a new Macherey Nagel column CC125/4 Nucleodur 100-5-C_{18}ec. The standards (15β-, 11α-, 9α- and 6β-hydroxyprogesterone) were checked for the retention time in the new system and compared with the old column from Nova Pak. On the new column, all the steroids had longer retention times than on the old column. 15β-, 11α-, 9α-, 6β-hydroxyprogesterone showed peaks at 4.91, 5.33, 6.23 and 6.93 min, respectively.

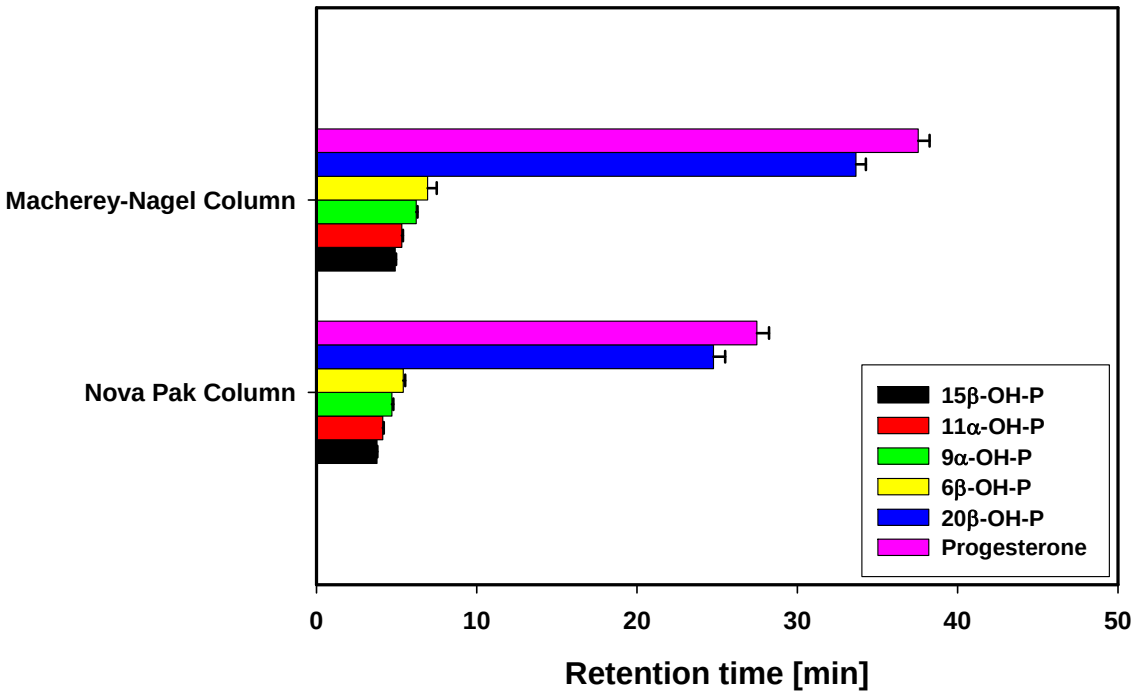


Figure 3.9: HPLC retention time of the hydroxylated progesterone products in 2 different columns, Nova Pak Nucleosil C_{18} (4 µm, 3.9 x 150 mm) and Macherey-Nagel CC125/4 Nucleodur 100-5-C_{18}ec. Black: 15β-hydroxyprogesterone, red: 11α-hydroxyprogesterone, green: 9α- hydroxyprogesterone, yellow: 6β-hydroxyprogesterone, blue: 20β-hydroxyprogesterone and pink: Progesterone.

3.3.2. Effect of solvent for progesterone conversion

The difference between the conversion *in vitro* and *in vivo* is working without or with living cells. Gram-negative *E.coli* bacteria cells are sensitive with the agents as a result of the effective permeability barrier function of their outer membrane. The outer membrane is impermeable to macromolecules and allows only limited diffusion of substances through its lipopolysaccharide covered surface (Vaara, 1992). Substances, which penetrate through the porin channels easily, have to be hydrophilic compounds or have to use some kind of carriers. For example, steroids are usually dissolved in ethanol or methanol. Since alcohol can be harmful to the cell, some substances which are hydrophobic or are larger hydrophilic compound, which penetrate the outer membrane poorly, need another type of a carrier such as cyclodextrin. To compare the effect of the solvent carrier on the cells and the resulting *in vivo* conversions, 10 mM progesterone was resolved in absolute ethanol (HPLC grade) and in 45% of hydroxypropyl-β-cyclodextrin (HPBCD). Two molecules of hydroxypropyl-β-cyclodextrin are able to carry a single molecule progesterone through the cell membrane and are removed afterwards (Vaara, 1992). CYP106A2 wild type and 3 mutants CYP106A2 T89N/A395I, CYP106A2 A106T/A395I, CYP106A2 A106T/A395I/R409L were investigated in both solvent carrier solutions. Interestingly, the conversions with ethanol were more efficient than those with the cyclodextrin carrier in all the tested cases. After 6 hr conversion, all the CYP106A2 proteins converted 98 - 99% protesterone in absolute ethanol, but only up to 38.5% progesterone consumption after 12 hr in cyclodextrin solution were reached (Figure 3.10).

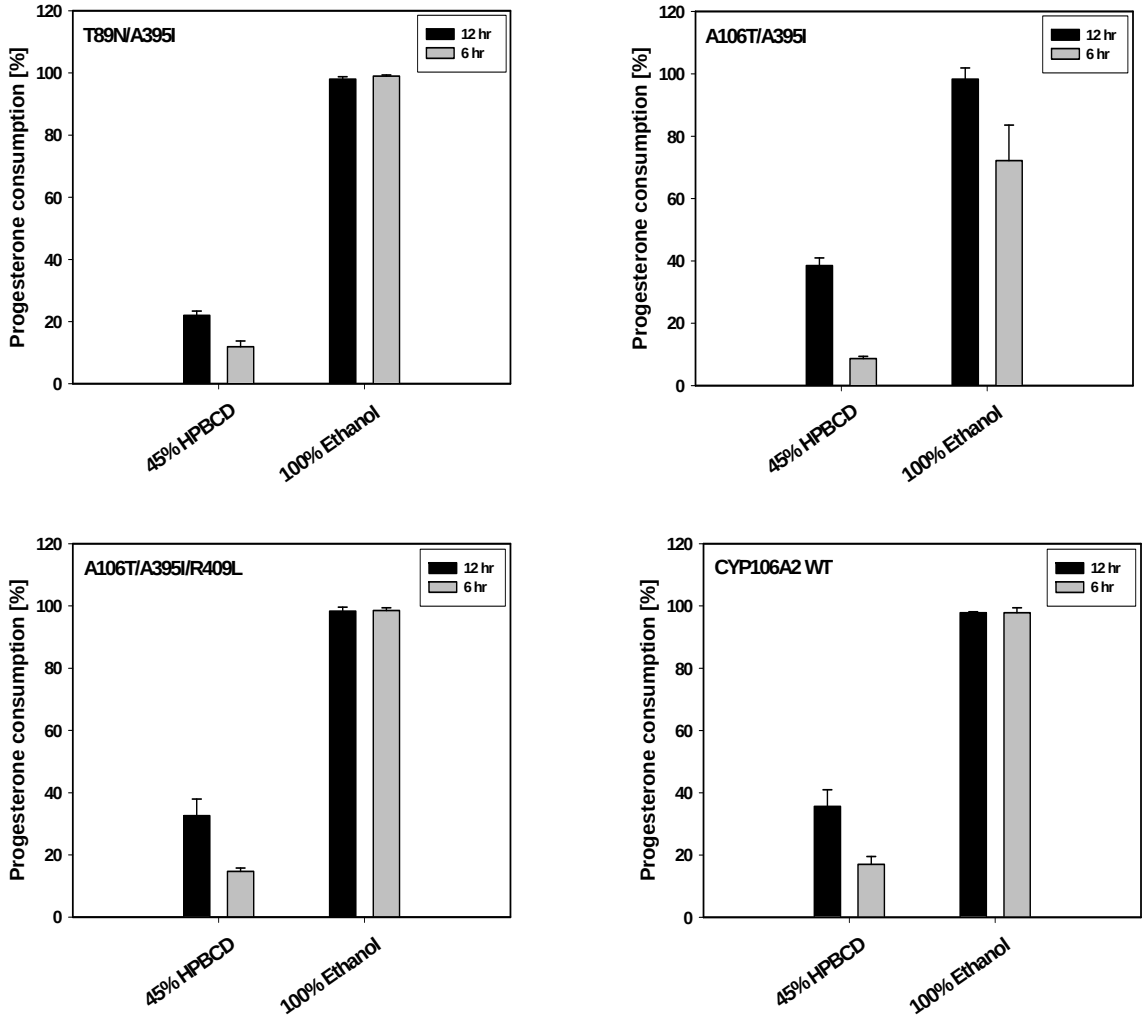


Figure 3.10: Consumption of progesterone by CYP106A2 wild type and 3 mutants (CYP106A2 T89N/A395I, CYP106A2 A106T/A395I, CYP106A2 A106T/A395I/R409L) in absolute ethanol and 45% Hydroxypropyl-β-cyclodextrin-carriers. Grey bars: conversion after 6 hr and black bars: conversion after 12 hr.

3.3.3. *In vivo* conversion of progesterone by selected mutants

CYP106A2 wild type and 7 chosen mutants were reconstituted with Adx and AdR from bovine adrenals by coexpression of pACYC_FHH2_CYP106A2 variants and pBAR_Twin in 20 ml of *E.coli* JM109 culture. The negative controls for every sample were transformed and expressed with only the plasmid pBAR_Twin. After 24 hr of induction, 1 ml of culture was taken out and washed with potassium buffer (pH 7.4) to avoid the impurity peaks when analyzing the products by HPLC. The cells were dissolved once in potassium buffer (pH 7.4) and 100 µM and/or 200 µM of progesterone was added to start the reaction at 30°C for 6 hr and 12 hr, respectively. Steroids were extracted with chloroform and applied to an HPLC column for analysis of the products (Figure 3.11, 3.12 and Table 3.11, 3.12).

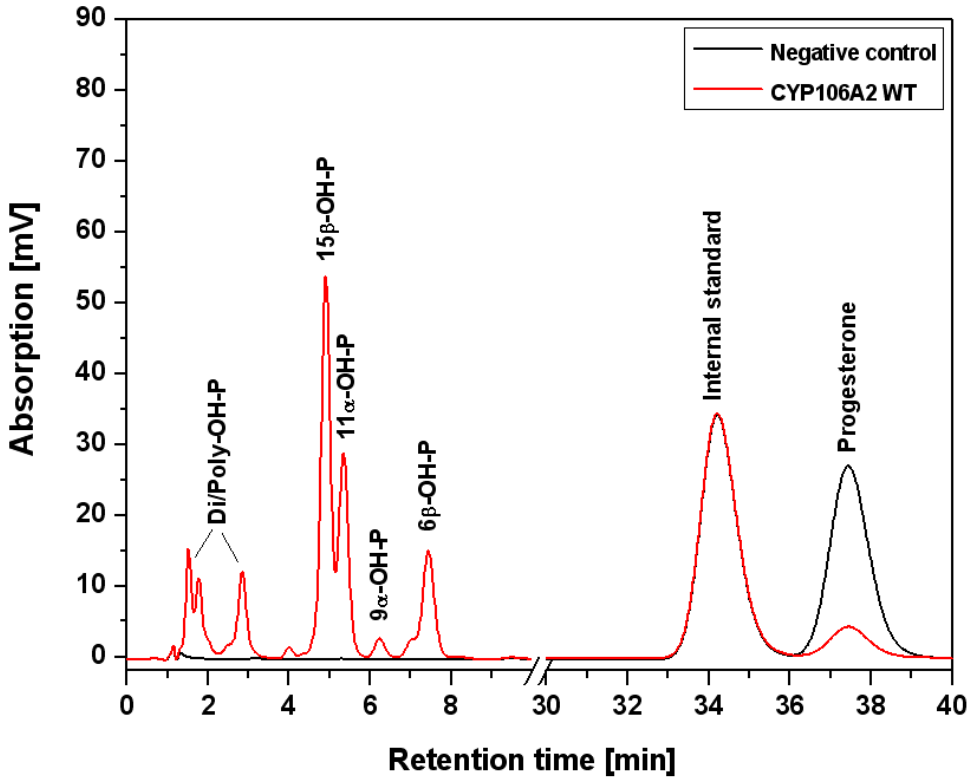
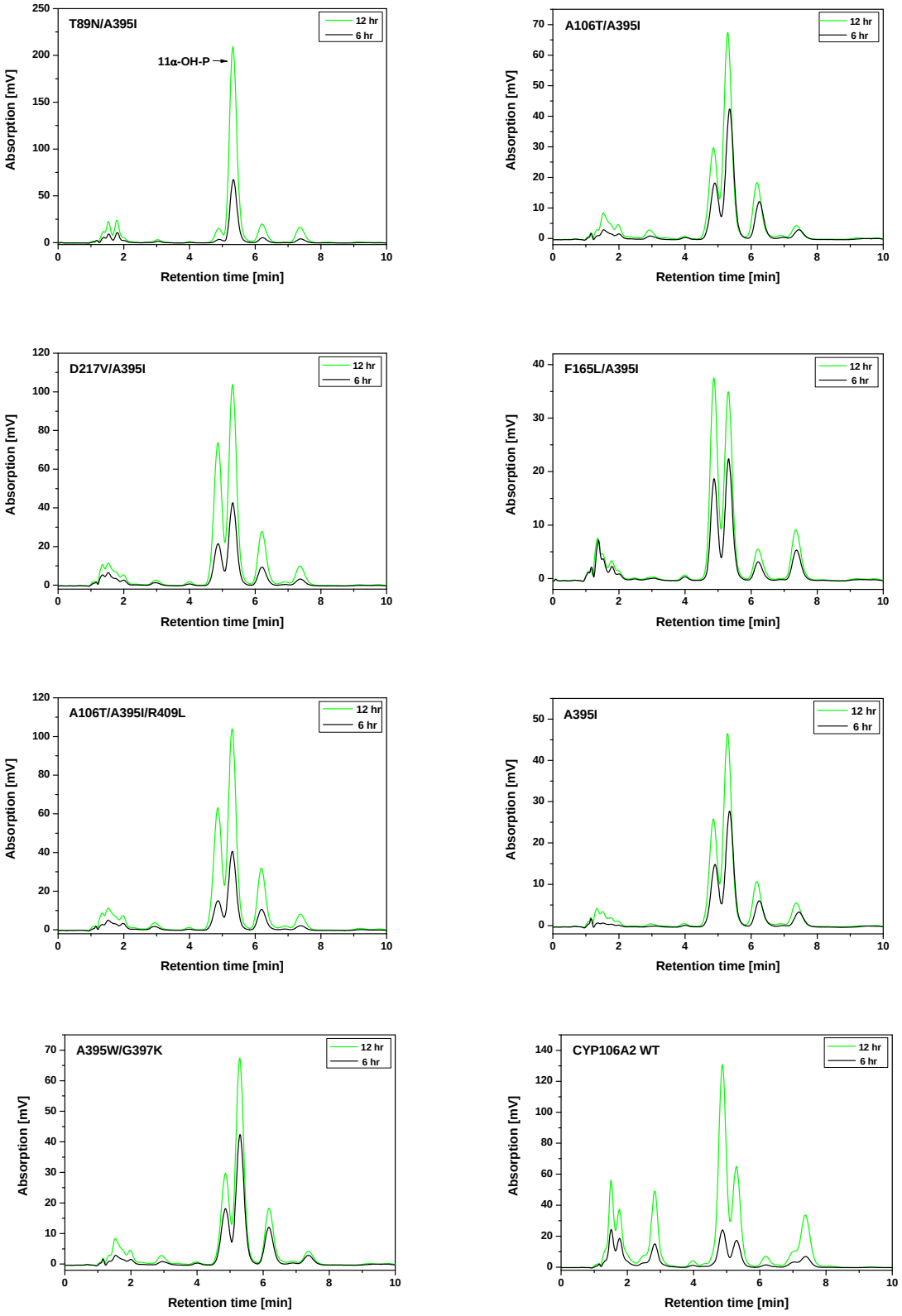


Figure 3.11: Chromatogram of hydroxylated progesterone products after 6 hr from CYP106A2 wild type (red line). The negative control (black line) was done the only single vector pBAR_Twin.



Firgure 3.12: Chromatograms of the hydroxylated progesterone products from CYP106A2 wild type and selected mutants in the whole-cell system. The plots after 6 and 12 hr conversion are in black and green, respectively.

Table 3.11: Distribution of hydroxylated progesterone products after 6 hr of *in vivo* conversion.

	15β-OH-P [%]	**11α-OH-P [%]**	**9α-OH-P [%]**	**6β-OH-P [%]**
T89N/A395I	100	1672.2 ± 95.2	161.5 ± 18.6	134.0 ± 16.1
A106T/A395I	100	227.5 ± 11.7	70.5 ± 4.1	20.3 ± 1.6
D217V/A395I	100	198.2 ± 17.0	48.5 ± 1.7	19.2 ± 0.6
F165L/A395I	100	127.3 ± 12.9	22.6 ± 0.8	28.3 ± 1.2
A106T/A395I/R409L	100	255.6 ± 14.3	74.3 ± 3.2	19.1 ± 0.4
A395I	100	186.7 ± 5.6	47.1 ± 1.3	28.9 ± 0.5
A395W/G397K	100	228.5 ± 13.4	68.1 ± 5.5	18.7 ± 0.9
CYP106A2 WT	100	73.0 ± 1.8	8.3 ± 0.02	35.1 ± 0.7

Table 3.12: Distribution of hydroxylated progesterone products after 12 hr of *in vivo* conversion.

	15β-OH-P [%]	**11α-OH-P [%]**	**9α-OH-P [%]**	**6β-OH-P [%]**
T89N/A395I	100	1436.3 ± 86.6	158.6 ± 15.1	139.4 ± 14.4
A106T/A395I	100	220.8 ± 19.5	66.3 ± 7.5	17.2 ± 1.7
D217V/A395I	100	142.3 ± 11.6	40.9 ± 1.1	16.1 ± 0.3
F165L/A395I	100	99.9 ± 1.4	18.8 ± 0.3	30.6 ± 1.6
A106T/A395I/R409L	100	161.2± 13.4	53.5 ± 4.6	14.7 ± 0.4
A395I	100	184.4 ± 8.4	47.3 ± 0.8	26.3 ± 0.6
A395W/G397K	100	188.5 ± 9.4	54.1 ± 1.3	24.7 ± 0.3
CYP106A2 WT	100	50.2 ± 1.8	7.0 ± 0.04	39.2 ± 0.9

In general, during *in vivo* conversion all the cases showed a higher amount of 11α-hydroxyprogesterone compared with the *in vitro* experiments except for mutants CYP106A2 A106T/A395I, CYP106A2 F165L/A395I and CYP106A2 A395W/G397K, which showed slightly less (5.2 - 18.5%) 11α-hydroxyprogesterone activity . The proportion of 15β/11α/9α/6β from *in vivo* conversion of CYP106A2 wild type was 100/73/8.3/35.1, which was different from the *in vitro* conversion (100/5/1.5/6.4). In mutant CYP106A2 F165L/A395I, the 11α-formation was not higher as compared with 15β-hydroxylated product formation; furthermore, after 12 hr, 11α-hydroxyprogesterone

production was lower than the 15β-hydroxyprogesterone production. Moreover, this mutant showed quite low activity in the whole-cell system compared with the *in vitro* conversion. The two parental mutants (CYP106A2 A395I and CYP106A2 A395W/G397K) still showed a rather low activity although the percentage of 11α-hydroxyprogesterone using CYP106A2 A395I was higher compared with the isolated enzyme of this mutant.

Clearly, the *in vivo* conversion after 6 hr produced more 11α-hydroxyprogesterone than after 12 hr, where the 15β-hydroxylation was increased in every CYP106A2 protein. After 12 hr, 15β-hydroxyprogesterone was increased as compared with the others products, 11α-, 9α- and 6β-hydroxyprogesterone.

Taken together, it can be stated that new mutants with changed selectivity and improved activity were obtained. The most successful mutant was CYP106A2 T89N/A395I, which exhibited mainly 11α-hydroxyprogesterone (1672.2% compared with 15β-hydroxylation) in the whole-cell system. Moreover, CYP106A2 T89N/A395I became the most interesting mutant for the 11α-hydroxyprogesterone production, as it also showed the highest conversion rate in comparison with the others. Moreover, mutant CYP106A2 A106T/A395I/R409L showed 255.6% of 11α-hydroxylation compared with 15β-hydroxylation as well as 95% progesterone consumption after 6 hr of *in vivo* conversion.

4. DISCUSSION

4.1. Progesterone bioconversion

The importance of microorganisms for the synthesis of steroid drugs has been anticipated in 1952 when the production of 11α-hydroxyprogesterone by a *Rhizopus* strain was patented (Murray and Peterson, 1952). Moreover, enzymes, which are able to hydroxylate progesterone, are derived from filamentous fungi. Similarly, the filamentous fungus *Nectria haematococca* strain 110 contains 4 enzymes that metabolize exogenous steroids. Two of these are microsomal cytochromes P450, which act sequentially on progesterone. Firstly, a side-chain cleavage enzyme converts progesterone into androstenedione. The C-19 steroid androstenedione then is converted into an 11α-hydroxylated derivative through an 11α-hydroxylase. Two other conversions may occur after side-chain cleavage. The enzyme 1-ene dehydrogenase is specific for androstenedione and leads to an unsaturated bond within the A ring. The C-17-specific oxidoreductase is also involved in the production of androstenedione and testosterone from progesterone (Ahmed *et al*., 1996). Furthermore, algae are also reported as steroid metabolizing organisms. Some species such as *Cyanidium emersonii*, *Muriella aurantiaca* and *Selenastrum capricornutum* catalyze the hydroxylation of progesterone. The first alga produces 2β-, 6β-, 9α-, 14α-, 16α- and 21-hydroxyprogesterone, while *M. aurantiaca* and *S. capricornutum* show as products only 6β-hydroxyprogesterone along with 15α-hydroxyprogesterone. The alga *Scenedesmus quadricauda* biotransforms progesterone into 9α-hydroxyprogesterone and 3-hydroxy-9,10-seco-pregna-1,3,5(10)-triene-9,20-dione (Pollio *et al*., 1994).

Bacterial enzymes hydroxylating progesterone are found in *Bacillus*, *Mycobacterium* and *Streptomyces* species. Some enzymes are able to convert progesterone into other intermediates. For example, *Mycobacterium smegmatis* possesses an enzyme which can produce progesterone from plant sterols derived from the pulpe of pine trees. It further converts progesterone into 17α-hydroxyprogesterone, androstenedione, and other androgens (Jenkins *et al*., 2004). Other enzymes such as Baeyer-Villiger monooxygenases (BVMO) also have the capacity of progesterone conversion. A fungal Baeyer-Villiger-steroid monooxygenase from *Cylindrocarpon radicicola*, esterifies progesterone to produce testosterone acetate and, subsequently, oxidatively lactonises in the next step the androstenedions to testololactone (Itagaki, 1986a and b). A bacterial Baeyer-Villiger monooxygenase from *Rhodococcus rhodochrous* also converts progesterone to

testosterone acetate (Miyamoto *et al*., 1995). Likely, cytochrome P450 from the *Streptomyces roseochromogenes* NCIB strain 10984 hydroxylases exogenous progesterone, firstly to 16α-hydroxyprogesterone and then to 2β, 16α-dihydroxyprogesterone (Berrie *et al*., 1999).

The bioconversion of progesterone by microorganisms, which is summarized in Table 4.1, exhibits a significant diversity not only in different species but also concerning the products of the progesterone derivatives. In general, microbial steroid conversion has a lot of advantages, which can be applied for the biotechnological purpose as the microbial enzymes meet the demands of directed evolution. Furthermore, bacterial cytochromes P450, being able to perform steroid hydroxylation, can be heterologously expressed in a soluble form and are, therefore, the potential candidates for biotechnological application.

Table 4.1: Progesterone conversion catalyzed by microbial systems (*part 1*).

Organism and Enzyme	Product	Reference
Bacillus cereus var. *mycoides* (MB 718) unknown	14α-OH-progesterone 11α-OH-progesterone	Mc-Aleer *et al.*, 1958
Bacillus megaterium ATCC 13368 15β-hydroxylase (CYP106A2)	15β-OH-progesterone 11α-OH-progesterone 9α-OH-progesterone 6β-OH-progesterone	Berg *et al*., 1976 Lisurek *et al*., 2004 Kang *et al*., 2004
Bacillus thermoglucosidasius strain 12060 cytochrome P450	6β-OH-progesterone 6α-OH-progesterone androstenedione	Sideso *et al*., 1998
Bacillus stearothermophilus cytochrome P450	20α-OH-progesterone 6β-OH-progesterone 6α-OH-progesterone 9,10-seco-20α-OH-progesterone 6β,20α-diOH-progesterone 6α,20α-diOH-progesterone 5α-pregnan-3,6,20-trione 6-dehydroprogesterone	Al-Awadi *et al*., 2001, 2002
Bacillus subtilus cytochrome P450	6β-OH-progesterone 6α-OH-progesterone 20α-OH-progesterone	Walaa *et al.*, 2009
Caldariella acidophila unknown	4-pregnen-3,6,20-trione 5α-pregnan-3,6,20-trione 20α-OH-4-pregnen-3-one 6β-OH-progesterone 6α-OH-progesterone	De Rosa *et al*., 1981
Mycobacterium smegmatis unknown	17α-OH-progesterone	Jenkins *et al*., 2004
Streptomyces roseochromogenes strain 10984 progesterone-16α-hydroxylase	16α-OH-progesterone 2β,16α-diOH-progesterone	Berrie *et al*., 1999
Pseudomonas sp. unknown	androsta-1,4-dien-3,17-dion 17β-hydroxyandrosta-1,4-dien-3-one	Dhar and Samanta, 1993

Table 4.1: Progesterone conversion catalyzed by microbial systems (*part 2*).

Organism and Enzyme	Product	Reference
Rhodococcus rhodochrous Baeyer-Villiger monooxygenase	testosteroneacetat	Miyamoto *et al*., 1995
Acremonium strictum PTTC 5282 unknown	15α-OH-progesterone 15α-OH-DOC 7β,15β-diOH-progesterone 6β,11α-diOH-progesterone 11α,15β-diOH-progesterone 6β,11α,17α-triOH-progesterone 11α,15β,17α-triOH-progesterone 7β,15β,17α-triOH-progesterone	Yoshihama, 1993 Faramarzi *et al.*, 2003
Aspergillus fumigatus cytochrome P450	11α-OH-progesterone 15β-OH-progesterone 7β-OH-progesterone 7β,15β-diOH-progesterone 11α,15β-diOH-progesterone	Smith *et al.*, 1994
Aspergillus ochraceus cytochrome P450	11α-OH-progesterone	Samanta and Ghost, 1987
Aspergillus niger cytochrome P450	11α-OH-progesterone 6β,11α-diOH-progesterone	Walaa *et al.*, 2009
Cochliobolus lunatus m 118 cytochrome P450	11β-OH-progesterone 11β,14α-OH-progesterone 7α,14α-diOH-progesterone 11-keto-14α-OH-progesterone	Vita *et al.*, 1994
Cylindrocarpon radiciola Baeyer-Villiger monooxygenase	testosteroneacetate	Itagaki, 1986
Fusarium culmorium unknown	15α-OH-progesterone 12β,15α-diOH-progesterone	Kolek *et al.*, 1998
Mortierella isabellina unknown	14α-OH-progesterone	Holland *et al.*, 1992
Nectria haematococca strain 110 C17-C20-lyase	androstenedione	Ahmed *et al.*, 1996
Penicillium decumbens ATCC 10436 unknown	5α-diOH-progesterone	Holland *et al.*, 1995

Table 4.1: Progesterone conversion catalyzed by microbial systems (*part 3*).

Organism and Enzyme	Product	Reference
Mucor piriformis 14α-hydroxylase	14α-OH-progesterone 5β,14α-diOH-progesterone 6β,14α-diOH-progesterone 7α,14α-diOH-progesterone 7β,14α-diOH-progesterone	Madyastha and Joseph, 1993 Madyastha, 1994 and 1996
Phycomyces blakesleeanus progesterone hydroxylase	7α-OH-progesterone 15β-OH-progesterone 6β-OH-progesterone 14α-OH-progesterone 15α-OH-progesterone	Smith *et al.*, 1991 Ahmed *et al.*, 1995
Rhizopus nigricans cytochrome P450	11α-OH-progesterone 6β,11α-diOH-progesterone 11α-OH-5α-pregnan-3,20-dione	Kim and Kim, 1991
Trichophyton mentagrophyton unknown	15α-OH-progesterone 11α-OH-progesterone 1-dehydroprogesterone	Clemons *et al.*, 1989
Curvularia clavata Jain unknown	7α,14α-dihydroxypregn-4-ene-3,20-dione 11β,14α-dihydroxypregn-4-ene-3,20-dione	Vujcić and Jankov, 1990
Chlorella emersonii strain C211-8b unknown	2β-OH-progesterone 6β-OH-progesterone 9α-OH-progesterone 14α-OH-progesterone 16α-OH-progesterone 21-OH-progesterone	Pollio *et al.*, 1994
Muriella aurantiaca strain C249-1 unknown	6β-OH-progesterone 15α-OH-progesterone	Pollio *et al.*, 1994
Selenastrum capricornutum strain T1648 unknown	6β-OH-progesterone 15α-OH-progesterone	Pollio *et al.*, 1994
Scenedesmus quadricauda strain T76 unknown	9α-OH-progesterone 3-hydroxy-9, 10-seco-pregna-1, 3, 5 (10)-triene-9, 20-dione	Pollio *et al.*, 1994

4.2. Expression and purification of CYP106A2

Bacillus megaterium ATCC 13368 was shown to be able to hydroxylate progesterone (Berg *et al.*, 1976). Later, a cytochrome P450 from this strain has been purified, which was able to perform 15β-hydroxylation of 3-oxo-Δ^4-steroids. However, the expression yield of the cytochrome P450, subsequently identified as CYP106A2, was only 8 pmol/mg of protein (Berg *et al*., 1979a).

Rauschenbach (1993) firstly cloned and completely sequenced the gene of this steroid 15β-monooxygenase (CYP106A2) and heterologously expressed it in *E.coli* and *B.subtilis* cells. The P450 concentration in crude protein extracts rose up to 77 - 260 pmol and 250 pmol per mg of total soluble protein in *E.coli* and *B.subtilis*, respectively (Rauschenbach *et al*., 1993). Subsequently, the cDNA of CYP106A2 was expressed in *E.coli* BL21DE3 using the expression vector pKKHC. This led to a significant increase of the expression yield up to 4400 pmol/mg of protein, corresponding to 4330 nmol/L culture (Simgen, 2000). The optimization of the expression conditions gave a yield of 8000 pmol/mg of total protein (corresponding to 13699 nmol/L culture) (Lisurek, 2004).

However, the expression level CYP106A2 using pACYC_FHH2 which is necessary to use when performing a coexpression of the redox partner with pBAR_Twin, was lowered significantly. The yield was only 1273 nmol/L culture, which was 10-fold lower in comparison with that using the pKKHC vector. Nevertheless, the expression system is sufficient enough to be used for the screening of the mutants and their subsequent purification. The highest content (1678 nmol/L culture) of cytochrome P450 was obtained with mutant CYP106A2 A106T/A395I. The other mutants varied in their expressed level from 400 - 800 nmol/L culture. It was found that only 8 CYP106A2 mutants (6, 19, 20, 21, 29, 40, 42, 43) showed lower expression levels (< 300 nmol/L culture). Although the yield of the expressed protein in pACYC_FHH2 system was lower than in other expression systems (e.g. using pKKHC as an expression vector), the pACYC_FHH2 plasmid has other advantages. E.g. it can be coexpressed with another plasmid (pBAR_Twin), which is providing the electron transfer partners AdR and Adx. This coexpression system is useful for the application of molecular evolution approaches in order to select mutants of CYP106A2 with higher stability, activity, and changed regio- and stereo-selectivity.

To get a reasonable amount of mutant proteins, the expression and purification of CYP106A2 had to be optimized. The heterologous expression of CYP106A2 was optimized using the single mutant CYP106A2 A395I and the double mutant CYP106A2 A395W/G397K. During the expression of the CYP106A2 mutants three factors (OD_{600}, pH and P450 content) were measured in parallel. Although the P450 content showed a difference between the two mutants (5.1 μM for CYP106A2 A395I and 3μM for CYP106A2 A395W/G397K), the OD_{600} reached approximately 3.0 after 32 - 44 hr of expression. During the investigated time, the pH value decreased from 7.4 to 6.6 in the first 20 hr and rose up to 7.0 again. However, in the mutant CYP106A2 A395I, the P450 content dramatically increased while the pH was at a minimum (6.6). In contrast, the amount of CYP106A2 increased steadily despite a reduced pH value in the double mutant (CYP106A2 A395W/G397K). In both cases, the maximum P450 content was gained at 44 hr of expression. This observation suggests that the heterologous expression of cytochrome P450 can be measured after 20 hr for the evaluation of a successful expression or to control other parameters such as pH or cell density during expression.

Several methods of cytochrome P450 purification procedures had been used in the past. Berg *et al.* purified CYP106A2 through 5 chromatographic columns including DEAE-cellulose, Ultrogel ACA-54, DEAE-Sepharose, octyl-Sepharose and HAP. During this complete purification procedure, approximately 97% of the cytochrome P450 was lost. In that case, 77.3% of P450 was lost during octyl-Sepharose and HAP columns (Berg *et al*., 1979a). Similarly, Simgen *et al.* purified CYP106A2 by $(NH_4)_2SO_4$ precipitation and then 3 chromatographic columns including DEAE-Sepharose, HAP and gel filtration. During this procedure, only 35 - 50% of the protein was lost (Simgen, 2000).

In this work, the purification procedure of Simgen was followed. The loss of the CYP106A2 content was about 50%. After applying on the DEAE Sepharose column, the loss of CYP106A2 was approximately 30 - 35% of cytochrome P450. The P450 content was reduced by 15 - 24% after application on the HAP column. Finally up to 10 - 15% was lost after gel filtration (Superdex 75). The absorptions of the protein at the wavelengths 417 nm and 280 nm showed the typical absorbance of a cytochrome P450 in its low-spin state. The ratio of A_{417}/A_{280} was used to monitor the purification level of the cytochrome P450. A higher ratio represented the more homogenous from of the CYP106A2. The ratio A_{417}/A_{280} of the CYP106A2 mutants was only around 1.0 - 1.2, being less than the value published before for the wild type enzyme (1.7 – 1.8) (Simgen,

2000; Lisurek, 2004). This finding could be due to more protein impurities or higher apo-protein levels probably caused by a decreased stability of the mutants.

4.3. Hydroxylation of progesterone by CYP106A2 mutants

4.3.1. Rational behind this study

CYP106A2 from *Bacillus megaterium* ATCC 13368 converts progesterone and produces not only 15β-hydroxyprogesterone but also 11α-, 9α- and 6β-hydroxylated products which rates ranged from 5 - 10% (Lisurek *et al*., 2004; Kang *et al*., 2004). Lisurek *et al.* constructed a homology model of CYP106A2 and identified 6 substrate recognition sites (Table 4.2) being important for the activity of this enzyme. The amino acid residues lying within each substrate recognition site are therefore promising candidates for site-directed mutagenesis. So, in this work, they were chosen to alter the regio-selectivity and the activity of CYP106A2.

Table 4.2: The substrate recognition sites of CYP106A2 derived from a computer model (Lisurek, 2004)

Substrate recognition site (SRS)	Residues
1. B/B'- and B'/C-loop	R66, E78, S81, E84, N87, I88, T89
2. F-Helix	L171, F175, F178
3. G-Helix	not available
4. I-Helix	R235, M238, L239, A243, T247, T248
5. β1-4	F289, N290, L291, K293, L294, R296
6. β4-1/β4-2-loop	A395, T396

The regio-specificity of the progesterone hydroxylation was evaluated by Lisurek (Lisurek, 2004; Lisurek *et al.*, 2008). During the docking of progesterone into CYP106A2, at least one hydrogen bond between the C-3 keto group of progesterone and the loop between residue A395 and residue T396 within the putative substrate recognition site (SRS) 6 was found and no other SRS was observed to be involved. Therefore, these residues were chosen for site-directed mutagenesis and mutants were created to change the hydroxylation pattern. On the other hand, to change the regio-selectivity from the 15- to 11-position, CYP106A2 was aligned with human CYP11B1 to identify the analogue residues between the 2 proteins in the SRS 6 region. The alignment between these 2 enzymes in the SRS 6 showed a high degree of diversity between amino acids 394 and

398 of CYP106A2 giving rise to the production of 5 mutants (CYP106A2 S394I, CYP106A2 A395L, CYP106A2 T396R, CYP106A2 G397P and CYP106A2 Q398S). Distinct changes in the progesterone hydroxylation pattern were observed for the 2 central mutants in SRS 6 (CYP106A2 A395L and CYP106A2 G397L), whereas mutations in position 394 and 398 had no effect for the regio-selectivity, and mutant CYP106A2 T396R was an inactive enzyme (Lisurek, 2004; Lisurek *et al.*, 2008). It was assumed that, only 2 residues, A395 and G397 of CYP106A2, could affect the regio-selectivity of progesterone hydroxylation. Therefore, saturation mutagenesis was used in following studies to produce a library of mutants with changes in positions 395 and 397 (Virus, 2006). However, only a few mutants had been analyzed in that work so that further investigations using this library were necessary to screen for mutants with changed regio-selectivity of the hydroxylation.

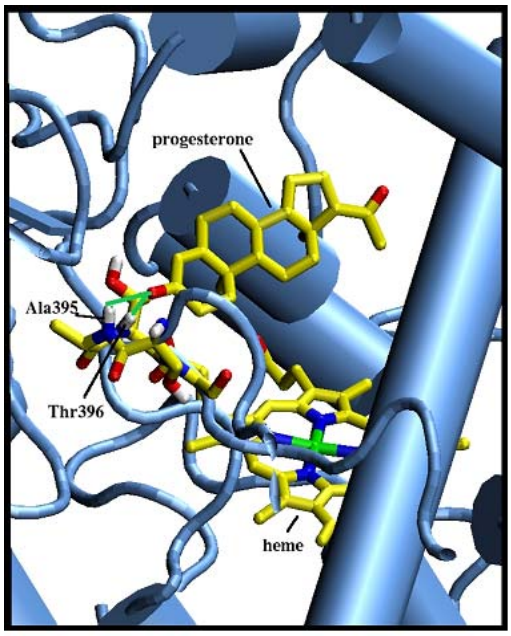


Figure 4.1: The binding pocket of CYP106A2 was docked with the substrate progesterone. The substrate, the heme-cofactor and the loop A395-G397 are shown in stick representation. The hydrogen bonds between the substrate and amino acid T396 are exhibited in green.

4.3.2. Progesterone hydroxylation of CYP106A2 mutants within the SRS 6 region

To screen for a changed regio-selectivity of hydroxylation, 40 transformants of the saturation mutagenesis library have been selected and studied for their product pattern. The CYP106A2 mutants were cloned into plasmid pACYC_FHH2 as described in section 2.2.4. Afterwards the mutants were expressed in *E.coli* JM109 and the corresponding proteins were purified. This work started with the aim to select a mutant which showed a

higher 11α-hydroxyprogesterone production rate. Therefore, after purification, the CYP106A2 mutant proteins were reconstituted with AdR and truncated Adx (4-108) for the *in vitro* studies (to convert progesterone). The hydroxylated products were analyzed using HPLC and it was found that the amount of the former side products (11α-, 9α-, 6β-hydroxyprogesterone) were higher in almost the active mutants compared with the CYP106A2 wild type where 15β-hydroxyprogesterone was the most prominent product. The active mutants showed at least 2-fold higher formation of the 11α-hydroxylated product than the wild type enzyme. Furthermore, 2 mutants (CYP106A2 A395K/G397N and CYP106A2 A395W/G397K) showed the highest proportion of 11α-hydroxyprogesterone being 13.5- and 56-fold, respectively, higher compared with the relative value of CYP106A2 wild type. Mutant CYP106A2 A395W/G397K produced more 11α-hydroxyprogesterone than 15β-hydroxyprogesterone (Table 4.2). Similarly, all the active mutants with high 11α-hydroxyprogesterone formation also showed at least 4-fold higher 9α-hydroxyprogesterone formation than that of CYP106A2 wild type. Mutant CYP106A2 A395R/G397K gave 125% of the 9α-hydroxylated product in comparison with 15β-hydroxyprogesterone (100%). However, this tendency was not observed for the hydroxylation at the C-6 position. In general, most of the mutants produced 2 - 4 folds more 6β-hydroxyprogesterone in comparison with CYP106A2 wild type, whereas mutant CYP106A2 A395H/G395L reduced the formation of the 6β-hydroxylated product. Taken together, the 2 SRS 6 mutants CYP106A2 A395W/G395K and CYP106A2 A395K/G397N were the most interesting ones showing a significant changes of the regio-selectivity towards the 11α-, 9α-, 6β-position (Table 4.2). Unfortunately, none of these most interesting mutants showed a similarly high turnover rate as CYP106A2 wild type.

Table 4.2: Relative regio-specificity towards 11α-hydroxyprogesterone formation of the CYP106A2 wild type and mutants. For each cytochrome, the 15β-hydroxyprogesterone amount was set to 100%. The obtained results are compared with the results of **1**: Lisurek (Lisurek, 2004), **2**: Virus (Virus, 2006) and **3**: Tin (Tin, 2006).

	15β-OH-P [%]	**11α-OH-P** [%]	**9α-OH-P** [%]	**6β-OH-P** [%]
CYP106A2-WT	100	5.0	1.5	6.4
A395K/G397N	100	67.7	33.2	63.8
A395W/G397K	100	282.2	69.6	107.7
CYP106A2-WT [1]	100	9.0	3.9	9.1
A395L [1]	100	38.9	20.0	7.0
G397P [1]	100	40.7	9.0	20.9
A395I [2]	100	97.2	32.1	13.5
A395L/G397S [2]	100	101.6	20.9	20.3
A395L/G397P [2]	100	61.3	25.3	13.5
A395I/G397D [3]	100	105.0	51.0	21.0
A395I/G397H [3]	100	73.0	39.0	0.0
A395F [3]	100	64.0	31.0	41.0

As shown in Table 4.2, the mutants with the so far highest formation of 11α-, 9α- or 6β-hydroxyprogesterone have been obtained in this work. The amount of 11α-hydroxyprogesterone was nearly 3-fold higher than the best value published so far, the amount of 9α-hydroxyprogesterone was 1.4-fold, and that of 6β-hydroxyprogesterone 5.2-fold higher. It can also be seen that, the ratio of 15β-hydroxyprogesterone to the other products (11α-, 9α- and 6β-hydroxyprogesterone; 100/5/1.5/6.4) was different from that described previously by Lisurek *et al.*, where the ratio was (100/9/3.9/9.1). This is due to a difference in the reaction time during the *in vitro* assay. The reaction time was set to 3 min in Lisurek's work (Lisurek, 2004), whereas in this study, 10 min were used. This shows that, the prolongation of the reaction time could cause changes in the hydroxylation pattern, leading to an increase of 15β-hydroxyprogesterone and a decrease of the other products (Table 4.2).

When analyzing the progesterone hydroxylation towards the 11α-position, a trend in altering residues at the positions 395 and 397 has been observed. In the CYP106A2 wild type, the residue in position 395 is an alanine, which is a non-polar amino acid and

occupies a small place in the structure of the enzyme. In the mutants which exhibited a higher ratio of 11α-hydroxyprogesterone, amino acid A395 was observed to change into a larger amino acid such as isoleucine, leucine, lysine, phenylalanine or tryptophan. Except for lysine, which is a polar amino acid, the others are uncharged and possess larger side chains than alanine. On the other hand, glycine in position 397 is also a small and non-polar amino acid. This residue was substituted mainly by polar amino acids such as asparagine, lysine, serine or aspartic acid, which are also larger than glycine. Interestingly, the combination between a non-polar amino acid in position 395 (isoleucine, leucine or tryptophan) and a polar amino acid (lysine, aspartic acid or serine) in position 397 resulted in the highest 11α-hydroxyprogesterone formation. In contrast, mutants CYP106A2 A395K/G397N or CYP106A2 A395L/G397P, where one of these positions was not occupied by an optimal amino acid, showed only 67.7% and 61.3% of 11α-hydroxyprogesterone, respectively. On the other hand, single mutants such as CYP106A2 A395F or CYP106A2 A395I were also affected showing an 11α-hydroxyprogesterone formation rose up to 64 and 97.2%, respectively. Furthermore, mutant CYP106A2 A395E/G397Stop hydroxylated progesterone as the CYP106A2 wild type enzyme showing 4 mono-hydroxylated products. Interestingly, despite having only 396 instead of 410 amino acids, this mutant worked properly. This raised the question about the role of the residues from position 397 to 410. These residues resided on the C-terminal part of the CYP106A2 sequence and probably might not play an important role for the progesterone hydroxylation. It was assumed that, the position 395 could be more important than position 397 to alter the regio-specificity.

In contrast, when investigating the progesterone hydroxylation at the 9α-position, no concerning distinct amino acids tendency could be observed. Mutant CYP106A2 A395R/G397K, showing 125.8% of relative 9α-hydroxyprogesterone formation, was altered at both positions to polar and positively-charged amino acids. On the other hand, mutant CYP106A2 A395E/G397V, showing 84.3% 9α-hydroxyprogesterone, was substituted by both a polar and non-polar amino acid. In mutant CYP106A2 A395W/G397K, producing 69.6% of 9α-hydroxy product, amino acid A395 was replaced by a non-polar amino acid, whereas amino acid G397 was changed to a polar, positively-charged one. When considering the formation of 6β-hydroxyprogesterone, it can be observed that, the mutants CYP106A2 A395R/G397K, CYP106A2 A395K/G397N, and CYP106A2 A395W/G397K showed an increased production of this product. This

suggests that, a combination of two polar, positively-charged amino acids or of a polar, positively-charged amino acid with a non-polar, neutral amino acid, could lead to an increase of the 6β-hydroxylation.

These results support the hypothesis that the free CYP106A2 enzyme may exist in a dynamic equilibrium with multiple conformations and that the equilibrium is apparently determined by a few critical residues including those at positions 395 and 397. As discussed by Joyce, due to the changing position of this equilibrium in the absence of a substrate, the mutations can decrease the proportion of the binding energy, which is used up in shifting the equilibrium on the substrate binding, and so leading to a net increase in affinity (Joyce *et al*., 2004).

It was shown that the pocket structure of P450 is geometrically flexible and can be altered by mutation of a single amino acid at a key position. For example, the substitution of phenylalanine by valine at position 209 of CYP2A5 altered the product specificity from the 2-position to the 7-position as the major hydroxylation site (Negishi *et al*., 1996b). Furthermore, mutations of amino acid residues in the substrate access and binding region may be evolutionarily fixed. They may provide the cytochrome P450 with new activities which are advantageous for the survival of an individual. The non-synonymous mutations were accumulated more within the substrate recognition sites than outside of these sites (Gotoh, 1992). Residue 478, which is located in the putative substrate recognition site (SRS) 6 of CYP2B2, has shown to play a role in controlling the stereo-selectivity of the androstenedione hydroxylation. It may be mediated by alterations in the secondary or tertiary structure of the cytochrome, leading to changes in the orientation of the substrate in the active site (Kedzie *et al*., 1991; He *et al*., 1992 and 1994). In CYP106A2, the both residues A395 and G397 lay on the putative SRS 6 and are small side-chain amino acids. The size of the side-chain may play a major role in determining the P450 activities (Iwasaki *et al*., 1991). Although the size is not the only factor, it remains to be understood, how a change of the size of the side-chain results in a dramatic alteration of the P450 activities (Negishi *et al*., 1996a). As mentioned before, CYP106A2 hydroxylates progesterone at 4 different positions. This suggests that the substrate can be fitted to 4 completely different orientations in the pocket. The substitutions of residues A395 and G397 by a range of larger amino acid residues may prevent the binding of the progesterone in the mode as seen in CYP106A2 wild type and directly result in the repacking and displacement of the structural unit.

However, the both mutants, CYP106A2 A395I and CYP106A2 A395W/G397K, which showed the highest 11α-hydroxyprogesterone production, had a 50% lower activity compared with CYP106A2 wild type. Therefore, these mutants were further mutated to improve their catalytic efficiency.

4.3.3. Progesterone hydroxylation of CYP106A2 mutants within the I-helix

In order to increase the turnover rate of the two selected mutants showing the highest 11α-hydroxyprogesterone formation, CYP106A2 A395I and CYP106A2 A395W/G397K, residues close to the central region of the heme, were chosen for replacement. The highly conserved threonine residue within the I-helix of the cytochrome P450 was previously found to play an important role for the electron transfer and the molecular oxygen activation. This residue facilitated the coordination of a water ligand to the ferric heme, the binding of oxygen to the ferrous prosthetic group, and the cleavage of the bound dioxygen molecule to provide the final activated oxidizing species. Therefore, mutation at this residue was reported to affect directly in catalytic efficiency of cytochromes P450 (Imai *et al*., 1989; Raag *et al*., 1991; Truan and Peterson, 1998; Kim *et al.*, 2008). According to the docked progesterone-CYP106A2 model, the residue T247 in the middle of the I-helix of CYP106A2 corresponded with the strongly conserved amino acid in most cytochromes P450. Residue T247 is placed in the active site adjacent to the heme and is followed by residue T248 (Figure 4.2). In order to verify their important role on the catalytic activity, the 2 mutants CYP106A2 A395I and CYP106A2 A395W/G397K were used as parental enzymes and the following mutations were introduced by site-directed mutagenesis: T247A, T247V, T247S, T247P, T247W, T247A and T248V.

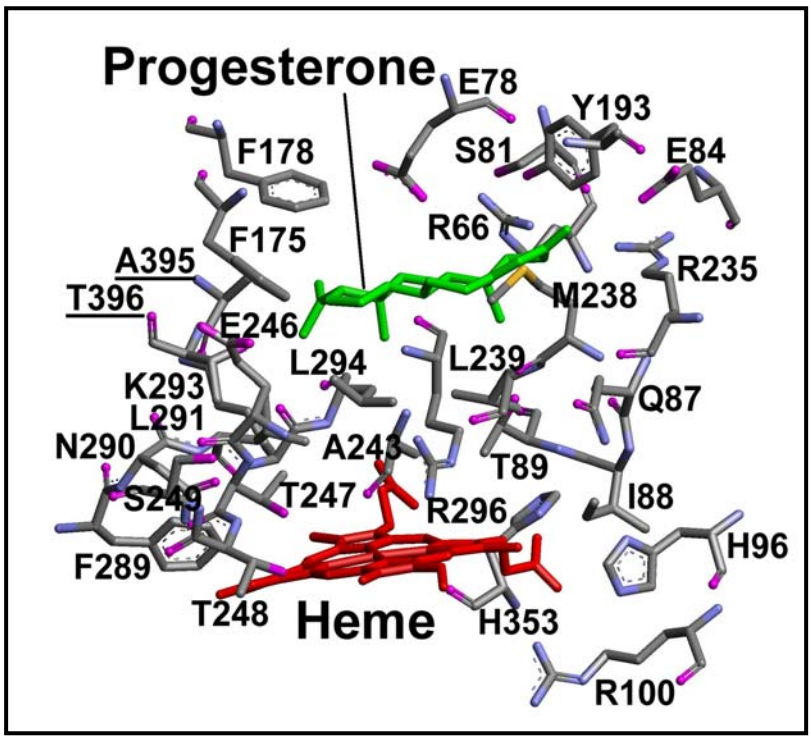


Figure 4.2: The residues forming the active site of the docked complex of CYP106A2 and progesterone. The heme is shown in red and the docked progesterone in green.

The results in this work revealed that the mutations at the positions T247 and T248 of CYP106A2 did not show a significant effect on the optical absorption of the enzyme. Even though, this could lead to subtle and local structural changes in the heme environment of the residues T247 and T248 as described for the analogous mutant CYP102A1 T252A (Raag *et al*., 1991). According to the docking experiment, residue T247 showed hydrogen bonds towards residues A243, V245, and T248, whereas residue T248 interacts with residues P147 and L252. The substitutions at positions T247 and T248 lead to an alteration of the hydrogen bond network in the active site. Besides the H-bond with residue V245, the mutation at residue T247 shifted the H-bond from residue A243 to residue S249 and to residue N290. In another case, the replacement of tryptophan for threonine in position 247 showed an additional H-bond with residue G242. Changing of residue T247 also showed to have an effect on the residue T248 surrounding area, where the connection with residue P147 was replaced by residues L251 and G364. Mutation at residue T248 created a new H-bond with residue H250 besides the connection with residues L251 and L252. Furthermore, the substitutions of residue T248 by alanine or valine also influenced the rearrangement of the H-bond network of residue T247 and its surroundings with residue N290. Moreover, the residue A243 showed a direct

interaction with progesterone. This resulted in a loss of the connection with residue T247. Consequently, residue N290 showed one H-bond directly to the progesterone, therefore, the substitutions of residues T247 and T248 may serve to alter the opening of a proton channel to the substrate binding pocket around the heme iron. It was shown that the replacement of the corresponding residue T243 from P450nor with asparagine, alanine and valine dramatically reduced the enzymatic activity (Obayashi *et al*., 2000), whereas the catalytic activity was considerably retained on substitutions by serine and glycine (Okamoto *et al*., 1998). Similarly, the mutations T214A, T214V, and T213A/T214A of CYP119 lead to a 2 – 3 fold increase of the styrene epoxidation activity compared to the CYP119 wild type (Koo *et al*., 2000 and 2002). In this study it was found that only 3 mutants (CYP106A2 T247V/A395I, CYP106A2 T247W/A395W/G397K and CYP106A2 T248V/A395I) showed a higher hydroxylation activity compared with the parental mutants (CYP106A2 A395I and CYP106A2 A395W/G397K) but still a lower activity than CYP106A2 wild type (Table 3.7). The estimations of enzyme kinetic parameters showed an improvement of the catalytic activity of these 3 mutants in comparison with that of CYP106A2 wild type. For the 15β-hydroxyprogesterone formation, the k_{cat}/K_m values of mutants CYP106A2 T247V/A395I, CYP106A2 T247W/A395W/G397K and CYP106A2 T248V/A395I were 4.87-, 4.98- and 2.87-fold, respectively, higher as that of CYP106A2 wild type. Interestingly, the 11α-hydroxyprogesterone formation also presented a higher turnover rate being 2.56-, 3.62- and 3.43-fold, respectively, higher as the 15β-progesterone formation rate of CYP106A2 wild type. Thus, the mutations at this conserved threonine residue may have an impact on the substrate recognition as well as on maintaining an efficiently functioning enzyme (Clark *et al*., 2006). The substrate recognition change was obviously followed by a change of the regio-selectivity of the hydroxylation. Unfortunately, only mutant CYP106A2 T248V/A395I retained a high proportion of 11α- hydroxyprogesterone (112.2%), as compared with 15β-hydroxylated product, while the others (CYP106A2 T247V/A395I, CYP106A2 T247W/A395W/G397K) lost their ability to hydroxylate predominantly at the C-11 position. The decrease in the progesterone regio-specificity may be related to the enzymatic turnover rate. In this case, the catalytic activity of CYP106A2 T247V/A395I, CYP106A2 T247W/A395W/G397K was faster than that of the parental mutants (CYP106A2 A395I and CYP106A2 A395W/G397K) which led to an equilibrium-disruption of the mutants in the substrate binding region placing an oxygen atom at the C-15 position. Nevertheless, in order to understand the role of this conserved threonine for

the correct positioning of the substrate and productive hydroxylation during the catalytic cycle of CYP106A2, the crystal structures of mutants and the wild type enzyme are necessary.

4.3.4. Progesterone hydroxylation of CYP106A2 mutants in other positions

Besides the conserved threonine in the I-helix, it was suggested that, another threonine at position 89 has an effect on the regio-specificity of the hydroxylation (Virus, 2006). The role of the corresponding residue was studied in CYP2C1, where residue V113 was replaced by an alanine. The mutant CYP2C1 V113A showed a 21-hydroxylase activity of progesterone, which was not observed in CYP2C1 wild type (Kronbach *et al*., 1991). Furthermore, it was shown that, the mutations V117A and F209L of the coumarin-7-hydroxylase (CYP2A5) were able to perform a 15α-testosterone hydroxylation (Lindberg and Negishi, 1989). Residue T89 of CYP106A2 corresponds to residue V113 of CYP2C1 as well as residue V117 of CYP2A5, whereas the residue F209 of CYP2A5 is related to residue F165 of CYP106A2. The amino acid T89 of CYP106A2 was first selected and mutated by Rauschenbach. However, the replacement of T89 by alanine, phenylalanine, and serine (mutants CYP106A2 T89A, CYP106A2 T89F and CYP106A2 T89S) did not show an increase in the catalytic activity of CYP106A2 towards 15β-hydroxyprogesterone formation (Rauschenbach, 1993). The evaluation of the regio-selectivity of progesterone hydroxylation by these mutants has not been done yet. On the other hand, if the residue T89 was substituted by an asparagine, which possesses a side-chain one methylene group longer than threonine and contains a more polar (amine) group, a dramatic increase of 11α-hydroxyprogesterone formation by 85- and 344-fold during the *in vitro* and *in vivo* conversion, respectively, compared with this formation in the wild type was observed (Tables 3.10, 3.11 and 3.12). Inspection of the computer model of CYP106A2 shows that a change of the H-bond network can be observed in mutant CYP106A2 T89N. Besides the H-bonds to the heme and residue R296, mutant CYP106A2 T89N also showed additional bonds to residues S64 and G87. The effect of this change is not fully understood so far, as it needs the crystal structure to verify changes of the substrate binding to this mutant. Besides this, substitution F209L of the coumarin-7-hydroxylase (CYP2A5) altered the regio-selectivity towards the testosterone 15α-hydroxylation (Lindberg and Negishi, 1989). The residue F165 of CYP106A2 corresponds with residue F209 of CYP2A5. However, this residue does not belong to the active center of CYP106A2. Therefore, a mutation at this position will probably not lead

to a change in the substrate regio-specificity. According to Virus's work, the mutant CYP106A2 F165I had no impact on the regio-selectivity of the progesterone hydroxylation. But this mutant as well as mutant CYP106A2 F165L showed increased catalytic efficiencies (1.25- and 2.5-fold) compared with CYP106A2 wild type (Rauschenbach, 1993; Virus, 2006). Interestingly, the apparent k_{cat}/K_m of 15β- and 11α-hydroxyprogesterone formations by mutant CYP106A2 F165L/A395I increased up to 9.0- and 10.3-fold, respectively, compared with the value of 15β-hydroxy product from CYP106A2 wild type, and up to 10.3- and 4.8-fold compared with the corresponding values for mutant CYP106A2 A395I.

The directed evolution of CYP106A2 to reach higher 11-deoxycortisol and progesterone hydroxylations by CYP106A2 resulted after one round of error prone PCR in 2 mutants, CYP106A2 A106T and CYP106A2 A106T/R409L, with an increment of 1.43- and 3-fold in case of progesterone and 11-deoxycortisol as a substrate, respectively. However, the single mutant CYP106A2 A106T seemed to be less stable than the double mutant CYP106A2 A106T/R409L. Therefore, the double mutant was chosen as a template for the second round of error prone PCR. The mutants CYP106A2 T89N/A106T/R409L and CYP106A2 A106T/Q189K/T399S/R409L presented an apparent k_{cat}/K_m of 15β-OH-RSS which were 3.3- and 4.3-fold higher than CYP106A2 wild type (Virus, 2006; Virus and Bernhardt, 2008). The combination of the substitutions A106T with the 2 selected SRS 6 mutants created a double (CYP106A2 A106T/A395I) and a triple mutant (CYP106A2 A106T/A395W/G397K). The new mutant CYP106A2 A106T/A395I exhibited a higher hydroxylation activity, whereas the triple mutant CYP106A2 A106T/A395W/G397K showed a similar activity as CYP106A2 wild type (Table 3.7). Investigation of the enzyme kinetics showed a 1.1-fold increase in the apparent k_{cat}/K_m of the 11α-hydroxyprogesterone formation with mutant CYP106A2 A106T/A395I and a 1.4-fold increase with mutant CYP106A2 A106T/A395W/G397K compared with the parental mutants (CYP106A2 A395I and CYP106A2 A395W/G397K). Moreover, the combination of mutations A395I and A395W/G397K with mutant CYP106A2 A106T/R409L led to an increase in the catalytic rate towards C-11 hydroxylation by a factor of 3 and 4.3, respectively. In contrast, mutant CYP106A2 T89N/A106T/A395I/R409L showed a decrease of the apparent k_{cat}/K_m compared with mutant CYP106A2 A395I. This decrease also influenced the distribution of the hydroxylated products. Interestingly, except for mutant CYP106A2

T89N/A106T/A395I/R409L, the other combinations still retained or even improved the regio-selectivity of progesterone toward the 11α-position. In comparison with the CYP101 structure of *Pseudomonas putida*, it was further suggested that residue A106 of CYP106A2 could have a critical function in the interaction with the redox partner adrenodoxin. Experimental evidence indicated that negatively-charged amino acids on the electron donor (Geren *et al*., 1984 and 1986; Bernhardt *et al*., 1984 and 1987) interact with positively-charged amino acids of the P450. A common region of positive charges is centered over the cystein pocket of all bacterial cytochromes P450 type I that interact with a ferredoxin (Stayton and Sligar, 1990; Wada and Waterman, 1992). The 3 arginine residues on the surface of CYP101 (R72 in the B-helix, R109 and R112 in the C-helix) are essential for the successful electron transfer from putidaredoxin (Pochapsky *et al*., 1996; Koga *et al*., 1993; Nakamura *et al*., 1994; Unno *et al*., 1996). The corresponding positively-charged residues in the CYP106A2 are K53, R97 and R100. Residue A106 is found in close proximity to the residues R97 and R100, in or next to the C-helix. The substitution of A106T from a non-polar amino acid to a polar amino acid could affect the binding of adrenodoxin to mutant CYP106A2 A106T. In contrast, mutation R409L did not seem to be involved in the interaction with adrenodoxin due to the far distance from the charged residues on the proximal site. According to the docking experiment, residue R409 did not belong to the active center of the enzyme and showed an H-bond with residues K377 and H378 in the β3-3 sheet, and with residue D131 in the β3-1 sheet. The replacement of R409 by a leucine leads to a change of the H-bond network. Residue L409 is connected to residues H378 and F376, which both are located in the β3-3 sheet. This mutation may create a minimal conformational change of the C-terminal part of CYP106A2 (Virus and Bernhardt, 2008).

In several P450 structures, the helices F and G form a flexible loop, which is part of the substrate access channel. This F/G loop will move towards the I-helix after substrate binding in order to close the access channel. This movement was observed in CYP101 (Dunn *et al*., 2001) and in CYP119 (Park *et al*., 2002). According to the docked progesterone-CYP106A2 model, residue D217 is located in the H-helix of the CYP106A2. This residue may be involved in the conformational changes process upon substrate binding (Virus and Bernhardt, 2008). A role of residue A243 in the steroid hydroxylation of CYP106A2 was also suggested by Virus (Virus, 2006). Although residue A243 directly contacted to progesterone in the docked progesterone-CYP106A2

model, its mutation (A243V) affected only the catalytic activity of the enzyme by increasing the 11-deoxycortisol hydroxylation 1.8-fold (Virus, 2006). This correlates with the effect observed with the mutant CYP106A2 A243V/A395I. It improved the apparent k_{cat}/K_m of 11α-hydroxylation 2.72-fold compared with the 15β-hydroxyprogesterone formation of CYP106A2 wild type due to a decrease of the K_m value.

4.4. *In vivo* conversion of progesterone

Biotransformation whole-cell systems are more popular than the isolated enzymes due to their economic advantages. Those systems can adapt to the pharmaceutical requirements for producing steroid intermediates, which are difficult to synthesize by chemical means. However, the most complicated point in the field of biocatalysts is cofactor-dependent redox reactions (Zhao and van der Donk, 2003; Urlacher *et al.,* 2004). Although the natural function of CYP106A2 is unclear, it requires an electron transfer system for its activity. In order to supply the reducing equivalents necessary for steroid hydroxylation activity, CYP106A2 was reconstituted *in vitro* with truncated Adx (4 – 8) and AdR from bovine adrenals. When using CYP106A2 *in vivo*, thus, also a corresponding redox chain must be available.

Directed evolution requires a fast screening system with a high accuracy. Hannemann *et al.* designed an *E.coli* system for whole-cell mediated steroid synthesis. The two-component electron transfer chain was implemented into *E.coli* cells by coexpression of the corresponding coding sequences from pBAR_Twin, containing the cDNAs of AdR and Adx together with plasmid pACYC_FHH2 containing the cDNA of CYP106A2 (Hannemann *et al.,* 2006). This system and the detection of the steroid hydroxylation by fluorescence measurement can be applied for screening (Appel *et al*., 2005; Virus *et al*., 2006). This combination meets the molecular evolution demands due to the reduction of time and costs as well as the reliability at measurements.

This method was validated in this work when the CYP106A2 wild type and the 32 mutant enzymes were coexpressed with pBAR_Twin and pACYC_FHH2, containing the cDNA of CYP106A2 or its mutants, in micro titer plates. From those experiments, 13 mutants were selected according to their higher relative fluorescence units compared with CYP106A2 wild type (Table 3.7). Eight mutants showed also a higher *in vitro* activity of progesterone hydroxylation (Table 3.9).

Besides using the whole-cell system for screening purposes, it can also be applied for steroid production. Interestingly, an alteration of the regio-selectivity of the *in vivo* progesterone hydroxylation was observed. The amount of 11α-hydroxyprogesterone in the whole-cell bioconversion increased compared to that of the isolated enzyme. Using the wild type protein, 11α-hydroxyprogesterone amounted to 5.0% and 73.0% in the *in vitro* and *in vivo* conversion, respectively, compared to the 15β-hydroxylation (set to 100%). The single mutant CYP106A2 A395I lead *in vivo* to 186.7% of 11α-hydroxyprogesterone, whereas *in vitro* conversion was only to 97.2%. The most dramatic change in the regio-specificity was achieved with mutant CYP106A2 T89N/A395I. This mutant showed a 4-fold higher 11α-hydroxyprogesterone production compared to 15β-hydroxyprogesterone formation. This ratio was raised up to 16-fold with the whole-cell biocatalyst system. An increase of the 11α-hydroxyprogesterone formation was also observed in mutant CYP106A2 D217V/A395I (from 114% to 198.2%). In contrast, the mutants CYP106A2 F165L/A395I and CYP106A2 A106T/A395I/R409L produced in both cases almost the same amount of 11α-hydroxyprogesterone, whereas the mutants CYP106A2 A106T/A395I and CYP106A2 A395W/G397K showed *in vivo* less 11α-hydroxyprogesterone production compared with the isolated enzyme. Furthermore, when the *in vivo* biotransformation was longer than 12 hr, the formation of 11α-hydroxyprogesterone decreased compared with the 15β-hydroxyprogesterone formation. The mutant CYP106A2 D217V/A395I reduced the 11α-hydroxyprogesterone formation from 198.2% to 142.3% and CYP106A2 A106T/A395I/R409L dropped the activity from 255.6% to 161.2%. *In vitro*, CYP106A2 hydroxylates progesterone mainly in the 15β-position. However, when a reconstitution system using CYP106A2 wild type not only with AdR and Adx but also with the alcohol dehydrogenase (ADH) from *Lactobacillus brevis* was performed, the main product of the progesterone conversion was 11α-hydroxyprogesterone, which was about 2-fold higher than the 15β-hydroxy product (Zehentgruber *et al*., 2010). This observation could be due to the effect of the cofactors in the electron transfer system or to the interaction between the proteins in the *E.coli* cells. The details of this mechanism still need to be investigated.

4.5. Outlook

Steroid derivatives obtained by microorganisms are often advantageously for the industrial synthesis of steroid drugs. So far, the 11*α*-, 11*β*-, 15*α*- and 16*α*-hydroxylations are accomplished in the steroid industry, mainly by microbial transformations (Jekkel *et al*., 1998). CYP106A2 exhibits a high potential for application in the biotechnological industry due to its ability to perform stereo- and regio-selective hydroxylations. This protein can be expressed with high yield in *E.coli*. By using directed mutagenesis, CYP106A2 mutants, which show an increased catalytic efficiency as well as a changed substrate regio-selectivity, were obtained. With regard to a possible biotechnological application, CYP106A2 should be studied with further substrates in order to determine its total substrate spectrum. On the other hand, the substrate spectrum and the hydroxylated products could also be expanded by using different, newly created CYP106A2 mutants.

In this work, the mutants CYP106A2 A106T/A395I and CYP106A2 A106T/A395I presented using the *in vitro* conversion a 2-fold higher portion of 11α-hydroxyprogesterone compared with 15β-hydroxyprogesterone. Interestingly, mutant CYP106A2 T89N/A395I produced *in vivo* mainly 11α-hydroxyprogesterone, which was 16-fold higher compared with the 15β-hydroxy product. 11α- and 11β-hydroxyprogesterone are reported to be inhibitors of the 11β-hydroxysteroid-dehydrogenase type I and II (Souness *et al*., 1995). The 11α-hydroxylation also plays a role in the production of key intermediates to synthesize Gestoden and Desogestrel, 2 widely used contraceptive drugs. The successful creation of numerousness mutants, which hydroxylate progesterone at the C-11 position, suggests that the same strategy can also be used to alter the regio-selectivity towards other positions, such as 6β, 7β, 9α, 11β, 14α or 15α. It should be noted in this context that 6β,14α-dihydroxyandrost-4-ene-3,17-dione and 14α-hydroxyandrost-4-ene-3,6,17-trione possess androgen activity and are useful inhibitors for breast cancer cells. The 7β-hydroxylation of finasteride can be useful a medicine for the prostate cancer therapy (Pamidi and Jia, 1998).

However, in order to apply the right strategy in altering the substrate specificity of CYP106A2, the crystallization of this enzyme and the determination of the crystal structure are necessary. The crystal structure will give a deeper insight into the structure-function relationship of this enzyme.

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6. APPENDIX

6.1. Media

6.1.1. Luria-Bertani (LB) Broth

Bacto-Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Add H_2O	1000 ml

6.1.2. Terrific Broth (TB)

Tryptone	12 g
Yeast extract	24 g
Glycerin	4 ml
Add H_2O	900 ml

After autoclaving, add 100 ml of sterilized potassium phosphate buffer

K_2HPO_4	0.72 M
KH_2PO_4	0.17 M

6.1.3. Trace element solution (140 µl/L Medium)

$FeCl_2$ x 6 H_2O	2.7 g
$CaCl_2$ x 2 H_2O	0.2 g
Na_2MoO_4 x 2 H_2O	0.2 g
$CoCl_2$ x 6 H_2O	0.2 g
$ZnCl_2$ x 4 H_2O	0.1 g
$CuSO_4$ x 5 H_2O	0.186 g
H_3BO_3	0.05 g
Add H_2O	90 ml

Salt mixture was respended in 10 ml of concentrated HCl and sterilized by filtration

6.1.4. SOC medium

Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
Add H_2O	980 ml

After autoclaving, add 20 ml of sterilized 1M glucose

6.2. Buffer

6.2.1. Preparation of chemically competent cells

6.2.1.1. Transfer buffer I (TFPI)

1M CH_3COOK:	3 ml
1M KCl:	10 ml
1M $CaCl_2$:	1 ml
Glycerol:	12 ml

Filled up to 80 ml with distillated water, adjusted pH to 6.1

Add 1M $MnCl_2$	5 ml

Filled up to 100 ml with distillated water, pH should be 5.8.

6.2.1.2. Transfer buffer II (TFPII)

0.2M MOPS:	1 ml
1M KCl:	0.2 ml
1M $CaCl_2$:	1.5 ml
Glycerol	2.4 ml

Filled up to 20 ml with distillated water, pH should be 6.5-7.0.

Both of TFPI and TFPII was sterilized by filtration and stored in the refrigerator at 4°C before used.

6.2.2. "Quick and Dirty" preparation

L1 buffer (pH 8.0):	100 μg/ml RNase A
	50 mM Tris/Cl
	10 mM EDTA
L2 buffer:	200 mM NaOH
	1% SDS
L3 buffer (pH 5.1)	2.8 M CH_3COOK

6.2.3. Purification of CYP106A2

Lysis buffer:	50 mM Tris/Cl, pH 8.5 1 mM EDTA 100 mM NaCl 0.1 mM DTE 1 mM PMSF
Buffer A:	20 mM potassium phosphate buffer pH 7.4 0.1 mM DTE
Buffer B:	Buffer A plus 0.5 M NaCl
Buffer C:	10 mM potassium phosphate buffer pH 7.4 0.1 mM DTE
Buffer D:	100 mM potassium phosphate buffer pH 7.4 0.1 mM DTE
Buffer E:	50 mM potassium phosphate buffer pH 7.4

6.3. Standard protein solutions vary from 20 - 2000 µg/ml

	x µl BSA standard (2000 µg/ml)	x µl Water/Buffer	Protein content (µg/ml)	Σ (µl)
A	100	0	2000	100
B	50	50	1000	100
C	37.5	62.5	750	100
D	25	75	500	100
E	20	140	250	160
F	40 of (E)	60	100	100
G	20 of (E)	80	20	100
H	0	100	0	100

6.4. Sodium dodecylsulphate (SDS) polyacrylamid gelelectrophoresis

6.4.1. Preparation of the polyacrylamide separating gel

Stock solutions	Final acrylamide concentration		
	10%	**12%**	**15%**
4xLT	3.75 ml	3.75 ml	3.75 ml
10% APS	75 µl	75 µl	75 µl
dest. H_2O	add 15 ml	add 15 ml	add 15 ml
30% AA/Bis	5 ml	6 ml	7.5 ml
TEMED	7.5 µl	7.5 µl	10 µl

6.4.2. Preparation of the polyacrylamide stacking gel

Stock solutions	5% acrylamide concentration
4xUT	2.5 ml
10% APS	50 µl
dest. H_2O	add 10 ml
30% Arcylamide/Biss	1.6 ml
TEMED	5 µl

6.4.3. Buffer

4x Buffer for separating gel (4xLT): 1.5M Tris/Cl, pH 8.8
0.4% SDS

4x Buffer for stacking gel (4xUT): 0.5M Tris/Cl, pH 6.8
0.4% SDS

2x SDS loading buffer: 125 mM Tris/Cl, pH 6.8
20% glycerol
4% SDS
10% β-mercaptoethanol
0.004% bromphenol blue

6.4.4. Staining solution: 0.1% Coomassie Brilliant Blue G-250
40% Methanol
10% Acetic acid

6.4.5. Destaining solution: 25% Methanol
10% Acetic acid

6.5. The DNA sequence of CYP106A2 (Rauschenbach *et al.*, 1993)

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ATGAAAGAAG TTATTGCAGT AAAAGAAATT ACTAGGTTTA AAACAAGGAC GGAGGAATTT        60
AGCCCGTACG CTTGGTGTAA AAGGATGTTA GAAAATGACC CTGTGAGTTA TCACGAAGGA       120
ACGGATACGT GGAATGTCTT TAAATATGAA GATGTGAAGC GGGTTCTCAG TGATTATAAA       180
CATTTTTCAA GTGTTCGGAA ACGGACGACG ATTTCAGTTG GAACGGATAG TGAGGAAGGT       240
TCTGTGCCTG AAAAGATCCA AATCACTGAA TCGGATCCAC CTGATCATAG AAAACGCCGT       300
TCACTGCTGG CAGCAGCATT CACACCTAGA AGTCTTCAAA ACTGGGAACC TCGCATTCAG       360
GAAATTGCAG ATGAATTGAT TGGACAAATG GATGGTGGAA CGGAAATCGA TATTGTGGCA       420
TCATTGGCGA GTCCGCTTCC GATCATTGTC ATGGCCGATT TGATGGGGGT TCCCTCGAAA       480
GATCGTTTAT TGTTTAAGAA ATGGGTGGAT ACCTTATTTC TTCCTTTTGA TAGAGAAAAG       540
CAAGAAGAAG TAGATAAATT GAAGCAAGTT GCAGCAAAAG AATACTATCA GTATTTGTAT       600
CCGATTGTTG TGCAAAAACG ATTGAACCCG GCGGATGATA TCATCTCAGA TCTATTGAAG       660
TCGGAAGTGG ATGGGGAAAT GTTTACGGAT GATGAGGTTG TCCGGACGAC CATGCTGATT       720
TTAGGTGCAG GAGTCGAGAC AACCAGTCAT TTATTGGCCA ATAGCTTTTA TTCGCTGCTA       780
TATGATGACA AAGAAGTTTA TCAAGAGTTA CATGAAAACC TGGATTTAGT TCCGCAGGCG       840
GTCGAAGAAA TGCTCCGTTT CCGATTCAAT CTTATTAAAT TGGATCGCAC TGTAAAGGAA       900
GATAACGATC TATTGGGAGT GGAATTGAAA GAAGGGGATA GCGTGGTTGT TTGGATGAGT       960
GCAGCTAATA TGGACGAAGA GATGTTTGAA GACCCCTTCA CACTTAATAT CCACCGCCCT      1020
AATAATAAGA AACATCTCAC ATTCGGTAAT GGCCCTCATT TCTGCCTCGG AGCACCGCTA      1080
GCCAGGCTGG AAGCGAAGAT TGCGCTTACT GCATTCCTGA AGAAATTCAA GCATATTGAA      1140
GCGGTGCCAT CGTTCCAGTT AGAAGAGAAT CTTACCGATT CAGCGACCGG TCAAACTTTG      1200
ACCTCACTAC CGCTTAAGGC AAGCCGCATG TAA                                   1233
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6.6. The protein sequence of CYP106A2

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XMKEVIAVKEITRFK TRTEEFSPYAWCKRM LENDPVSYHEGTDTW NVFKYEDVKR      55
VLSDYKHFSSVRKRT TISVGTDSEEGSVPE KIQITESDPPDHRKR RSLLAAAFTP     110
RSLQNWEPRIQEIAD ELIGQMDGGTEIDIV ASLASPLPIIVMADL MGVPSKDRLL     165
FKKWVDTLFLPFDRE KQEEVDKLKQVAAKE YYQYLYPIVVQKRLN PADDIISDLL     220
KSEVDGEMFTDDEVV RTTMLILGAGVETTS HLLANSFYSLLYDDK EVYQELHENL     275
DLVPQAVEEMLRFRF NLIKLDRTVKEDNDL LGVELKEGDSVVVWM SAANMDEEMF     330
EDPFTLNIHRPNNKK HLTFGNGPHFCLGAP LARLEAKIALTAFLK KFKHIEAVPS     385
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6.7 Publication resulting from this work

Kim Thoa Nguyen, Cornelia Virus, Michael Lisurek, Frank Hannemann, Rita Bernhardt (2007). Molecular Evolution of CYP106A2 from *Bacillus megaterium* ATCC 13368. Poster presentation. *Bio-Hanoi 2007* - Conference "From Biosciences to Biotechnology and Bioindustry", Hanoi (Vietnam).

Kim Thoa Nguyen, Cornelia Virus, Frank Hannemann, Rita Bernhardt (in preparation). Changing the regio-selectivity of CYP106A2 from C-15 to C-11 of progesterone.

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