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The Fascinating World of Steroids: *S. cerevisiae* as a Model Organism for the Study of Hydrocortisone Biosynthesis

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Introduction

The purpose of this review is to introduce the reader to the steroid world through an example of metabolic engineering consisting of the transfer of a mammalian biosynthetic pathway into a recombinant organism. It attempts to collect together the knowledge that has been accumulated around the mammalian steroid pathway with the view of building an efficient model for understanding its regulation.

Steroids are ubiquitous molecules present in most organisms. In mammals, plants, and fungi they are synthesized from acetylCoA, while in other organisms, such as

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Abbreviations: ACAT, acyl coenzyme A cholesterol acyltransferase; ADR, adrenodoxin reductase; ADX, adrenodoxin; aldosterone, (11β)-11,21-dihydroxy-3,20 dioxo pregn-4-en-18-al; ARE, acyl coenzyme A:cholesteryl acyltransferase-related enzyme; ARH1, ADR related homologue 1; ATF2, alcohol *O*-acetyltransferase; brassicasterol, ergosta-5,22-dienol; campesterol, ergosta-5-enol; cholesterol, cholest-5-en-3βol; corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; cortisone, 17α,21-trihydroxy-4-pregnene-3, 11,20-trione, 17-hydroxy-11dehydrocorticosterone; CPR, NADPH cytochrome P450 oxidoreductase; *CYC1*, the gene encoding cytochrome-*c* isoform 1; CYP11A1, cytochrome P450 steroid side-chain cleaving; CYP11B1, 11β-steroid hydroxylase; CYP17A1, 17α-steroid hydroxylase; CYP21A1, 21-steroid hydroxylase; Cyt-*b5*, cytochrome *b5*; 11-deoxycortisol, cortexolone or 17α,21-dihydroxyprogesterone; DHEA, dehydroepiandrosterone; ER, endoplasmatic reticulum; ergosterol, ergosta-5,7,22-triene-3βol; Erg2p, sterol C8-C7 isomerase; Erg3p, C-5 sterol desaturase enzyme; Erg4p, sterol C-24 (28) reductase; Erg5p, Δ 22(23) sterol desaturase; Erg6p, S-adenosyl methionine Δ-24-sterol-C-methyl-transferase; Gcy1p and Ypr1p, aldo-keto reductases; 3β-HSD, 3β-hydroxy steroid dehydrogenase/isomerase; hydrocortisone, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione or 11β,17α,21-trihydroxyprogesterone; LXR, ligand X receptor; M, molar concentration (mol. l^{-1}); mat, mature form of protein; NCP1, *S. cerevisiae* CPR; *PGK1*, the yeast gene encoding phosphoglycerate kinase; PM, plasma membrane; Δ7-Red, sterol Δ7 reductase; SCC, side-chain cleavage; sitosterol, 24β-ethyl-Δ5-cholesten-3βol; StAR, steroidogenic acute regulatory protein; *TDH3*, the yeast gene encoding glyceraldehyde-3-phosphate dehydrogenase 3; *TEF1*, the yeast gene encoding transcription elongation factor 1; YAH1, yeast ADX homologue 1.

Biotechnology and Genetic Engineering Reviews – Vol. 22, January 2006
0264–8725/06/22/213–252 $20.00 + $0.00 © Lavoisier/Intercept, 14 rue de Provigny, F-94236 Cachan cedex, France

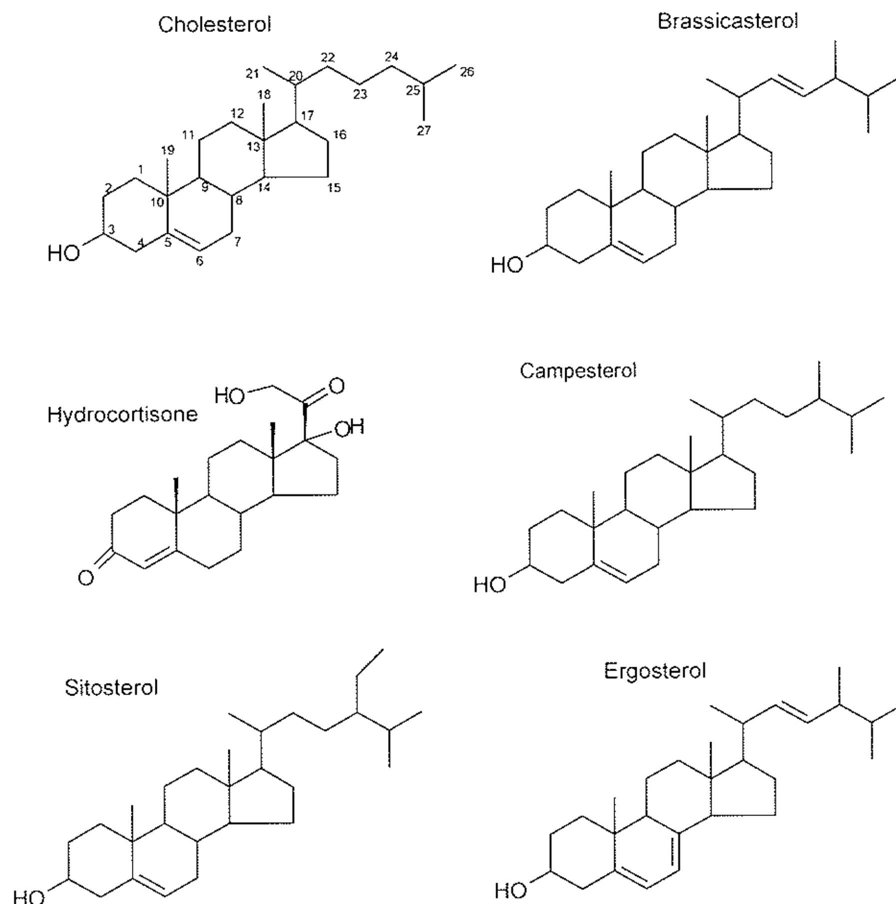


Figure 11.1. Cholesterol (1) planar structure and numbering. Planar structure of: brassicasterol; campesterol; hydrocortisone; sitosterol; and ergosterol.

insects or crustaceans, the external environment provides them. Around a common backbone of four cycles (*Figure 11.1*), nature has built a wealth of molecules that are fundamental for life. Through modifications of the steroid backbone by enzymatic reactions, such as dehydrogenation, aromatisation, hydroxylation, desaturation, and esterification, molecules have been created with distinct biological activities. In mammals, a side-cleaving enzyme transforms cholesterol into pregnenolone, which is the first precursor of steroid hormones. Pregnenolone is then metabolized by a small set of enzymes into three kinds of steroids: glucocorticoids, mineralocorticoids, and sex steroids. They are produced in specialized tissues, the adrenal cortex with different functional compartments, the gonads, and the placenta, and act through specific nuclear or membrane-bound steroid receptors (Norman *et al.*, 2004). In humans, hydrocortisone (cortisol) is the main glucocorticoid and aldosterone the main mineralocorticoid. The balance in mammalian plasma between the abundant hydrocortisone (160–50 ng/ml) and, for example, aldosterone (0.07 ng/ml) is of fundamental importance for normal development and differentiation of cells (as reviewed in Lisurek and Bernhardt, 2004). Pregnenolone, progesterone, and hydro-

cortisone are the most abundant steroids of the plasma derived from cholesterol. In other words, the hydrocortisone biosynthesis pathway produces steroid hormones efficiently, quickly, and precisely.

As a drug, hydrocortisone has a weak glucocorticoid effect and it is the starting material of choice for the chemical synthesis of drugs with potent anti-inflammatory, abortive, or anti-proliferative effects. In 1952, Woodward and colleagues reported the total synthesis of hydrocortisone in about 40 steps (Woodward *et al.*, 1952). Currently, a rather sophisticated, multi-step chemical procedure including a bioconversion by a natural microorganism is employed for the industrial manufacture of hydrocortisone starting from naturally occurring sterols. A continuous demand exists for finding new and innovative synthetic routes. A process mimicking the mammalian pathway in a recombinant microorganism might meet these expectations.

Besides its obvious economical relevance, the engineered system could help in our understanding of the complex pattern of cross-talk involved in this pathway. These engineered strains could permit the study of the connection and interactions between the different enzymes and membranes.

In mammals, synthesis of hydrocortisone proceeds through five enzymatic steps requiring eight proteins, five of which are membrane-bound enzymes. Included in this pathway are four members of the P450 superfamily of monooxygenases and the 3β-hydroxy steroid dehydrogenase/isomerase (3β-HSD), and three are electron carriers (Miller, 1988). The first reaction after cholesterol insertion into the inner mitochondrial membrane is its side-chain cleaving generating pregnenolone. This reaction is catalysed by CYP11A1 (Zuber *et al.*, 1988) and its companion electron carriers, adrenodoxin (ADX), a soluble component of the mitochondrial matrix (Vickery, 1997; Grinberg *et al.*, 2000), and adrenodoxin reductase (ADR), loosely bound to the inner mitochondrial membrane (Hiwatashi and Ichikawa, 1982) (*Figure 11.2*). Pregnenolone is metabolized into progesterone by 3β-HSD (Payne *et al.*, 1997), then hydroxylated twice in the endoplasmatic reticulum at positions 17 and 21 by corresponding P450s and the associated NADPH P450 oxidoreductase (CPR) (Yasukochi and Masters, 1976; Miller, 1988). Finally, the resulting product, 11-deoxycortisol, is transferred to mitochondria, where it is hydroxylated into hydrocortisone by CYP11B1 with the same electron carriers as CYP11A1 (Erdmann *et al.*, 1995).

In the first part of this review, we will give a brief introduction to the key steroids involved in the pathway. The natural hydrocortisone pathway and its specific features of concern in metabolic engineering will be presented in the second part. An emphasis will be made on the mitochondrial part of the pathway. The third part will be dedicated to *Saccharomyces cerevisiae* as a model organism used to synthesize steroids and to study the enzymes involved. The transfer of the pathway itself will be described, together with the hurdles associated with such an approach.

Key steroids in mammals, yeast, and plants

Sterols and steroids are fascinating molecules because of their presence in most living organisms. Built around a common backbone consisting of four rings and an aliphatic side-chain (*Figure 11.1*), a diversity of function and action has been achieved for mammals, plants, and yeast.

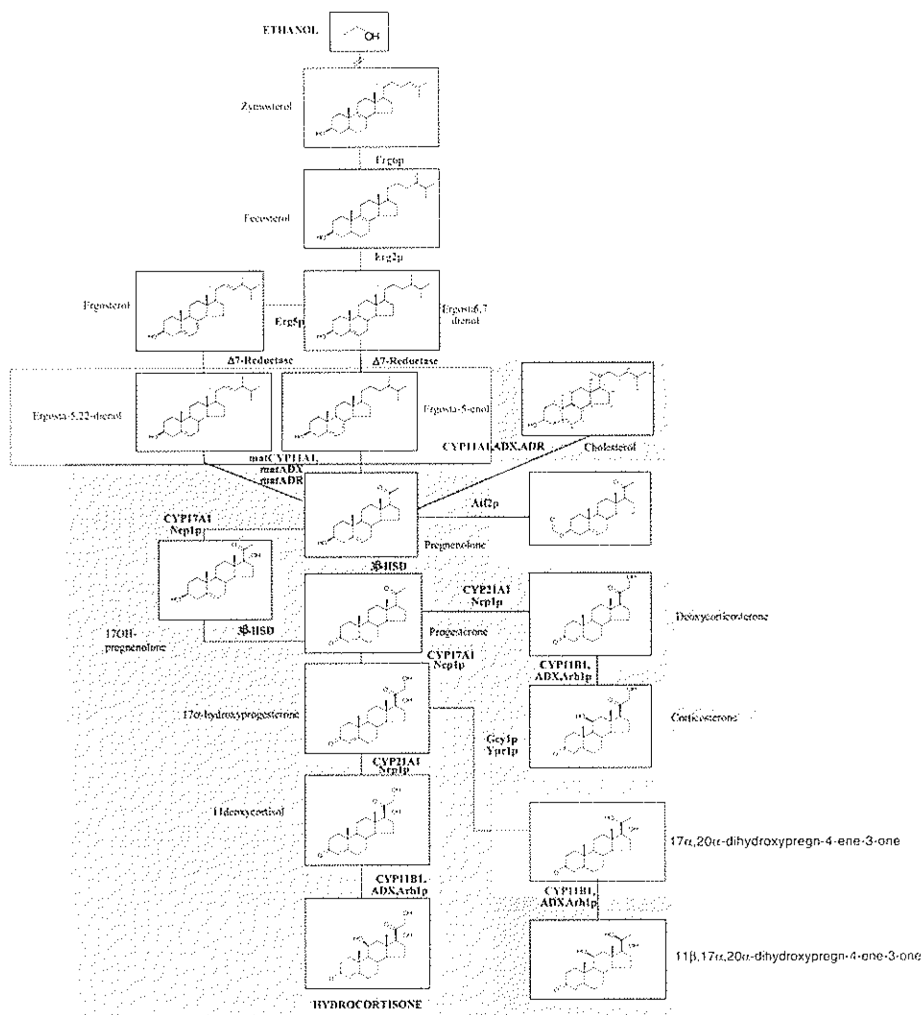


Figure 11.2. Schematic view of the engineered pathway linking the yeast sterol pathway to the mammalian hydrocortisone pathway. Connection of the ergosterol pathway with the hydrocortisone pathway. Ncp1p, NADPH P450 reductase; ADX, adrenodoxin; ADR, adrenodoxin reductase; Arh1p, adrenodoxin reductase related homologue; Atf2p, alcohol O-acetyltransferase (acetyl pregnenolone acetyl transferase); CYP11A1, P450 side-chain cleaving; CYP17A1, 17α-steroid hydroxylase; CYP21A1, 21-steroid hydroxylase; CYP11B1, 11β-steroid hydroxylase; Erg2p, sterol C8–C7 isomerase; Erg6p, S-adenosyl methionine Δ-24-sterol-C-methyl-transferase; Erg5p, Δ 22(23) sterol desaturase; 3β-HSD, 3β-hydroxy steroid dehydrogenase/isomerase; Gcy1p and Ypr1p, aldo-keto reductases. Legend: yeast sterols (light grey), steroids (grey), yeast-made derivatives from steroids (white dotted), Δ7-reductase products (white), side-product made by steroidogenic enzymes (dark grey), yeast endogenous proteins are marked as the respective gene products.

In these organisms, the squalene (C_{30}) is the early molecule that leads to triterpene compounds, whose cholesterol, ergosterol, campesterol, brassicasterol, and sitosterol, respectively, are major primary metabolites.

These sterols (*Figure 11.1*) form major components of plasma membranes. They

influence their fluidity and permeability, play a crucial role in membrane function and, for example, in the proper folding of membrane-embedded proteins (Umebayashi and Nakano, 2003; Troost *et al.*, 2004). In part, these sterols are also stored in specialized cellular organelles called lipid droplets or lipid particles, which serve as energy storage entities.

Finally, cholesterol, ergosterol, campesterol, brassicasterol, and sitosterol (*Figure 11.1*) have been shown to have minor structural differences. Ergosterol differs from the other sterols with the presence of a 7(8) double bond in ring B that rigidifies the backbone. All the other differences are situated on the side-chain in position 24, where a methyl can be found on ergosterol, campesterol, and brassicasterol, while an ethyl group is present in sitosterol. A double bond at position 22(23) is also present in ergosterol and brassicasterol. Cholesterol has a simpler side-chain with no methyl at position 24 and no double bond at position 22. While in yeasts, such as *S. cerevisiae*, ergosterol is the final metabolite of the pathway, in plants and mammals, the corresponding sterols are the starting entity for sterol and steroid hormone synthesis that is essential for the life of a multicellular organism.

In this review, we will describe a way of forcing yeast to synthesize plant sterols that can serve as a substrate for a mammalian P450, therefore permitting the production of mammalian sterols, such as pregnenolone.

From cholesterol to hydrocortisone in five steps: the hydrocortisone pathway

CHOLESTEROL TRANSPORT TO THE INNER MITOCHONDRIAL MEMBRANE

The reader is referred to *Figure 11.2*. It is not the purpose of this review to provide an outline of how cholesterol is synthesized or taken up by the cell before being transported to specialized sites for further metabolism. Instead, we will focus on the routing of yeast sterol synthesis to make mammalian steroids. A few examples of striking proteins involved in cholesterol transport will be given.

Whether supplied by low-density proteins, high-density lipoproteins, or *de novo* synthesis, cholesterol is the starting entity for the production of steroid hormones, and this has been well reviewed in Miller (1988). Cholesterol can be detected in both a free form and in an esterified form. The balance between these two forms varies dramatically from one cell type to another. Esterification takes place mostly on the hydroxyl moiety at position 3 of sterols and steroids, such as cholesterol, pregnenolone, estradiol, ergosterol (Mullner and Daum, 2004), and mollusc sterol (Janer *et al.*, 2004). The esterified sterols are stored in lipid droplets as a basic energy storage process, or they are exported. In principle, there is a continuous equilibrium between sterol esterified and non-esterified forms for rapid mobilization. Esters can be as different as acetyl, sulfatyl, or long-chain fatty acids, such as arachidonyl, palmytoleoyl, linoleoyl, oleoyl, and stearoyl. In mammals, cholesterol is esterified by acylcoenzyme A cholesterol acyl transferase (ACAT), a molecule that has been isolated and identified by Chang and co-workers (Chang *et al.*, 1993). More recent work has revealed the presence of a second ACAT protein (ACAT2), which has some redundancy with ACAT1 activity, although it is apparently expressed differentially (Oelkers *et al.*, 1998). ACAT1 and ACAT2 are deeply involved in cholesterol homeostasis: they are probably regulating the balance between

esterified cholesterol routed to lipid droplets or to lipoproteins (Buhman *et al.*, 2001).

The conversion of cholesterol into pregnenolone (*Figure 11.2*) by CYP11A1 takes place in the inner mitochondrial membrane (Shikita and Hall, 1973; Ishimura *et al.*, 1985; Ben-David and Shemesh, 1990) of producing cells. Exactly how the substrate is delivered to the inner mitochondrial membrane has been the subject of intensive research. At least two groups of proteins are now known to be involved in the intracellular trafficking of sterols, namely the NPC (Niemann–Pick type C) and START proteins. Both families have been identified with the help of two known genetic diseases, namely Niemann–Pick type C disease and congenital lipoid adrenal hyperplasia (lipoid CAH). The NPC disease is a lysosomal lipid storage disorder, and the lipoid CAH is a cholesterol transport and storage disorder. Deficiency of either of the two NPC1 and NPC2 proteins leads to the accumulation of non-esterified lipids (Cruz and Chang, 2000; Cruz *et al.*, 2000; Millard *et al.*, 2000). The precise mechanism of action of NPC1 and NPC2 is not understood but they are known to facilitate the intracellular transport of lipids between organelles and, in particular, lysosomes. NPC1 functions with a sterol-sensing domain and almost no binding affinity for sterols *in vivo* (Ohgami *et al.*, 2004). By contrast, NPC2 has been found to have a high affinity for cholesterol *in vivo* (Naureckiene *et al.*, 2000; Ohgami *et al.*, 2004).

Steroidic acute regulatory protein (StAR) is the prototype of the START (StAR related lipid transfer protein) family (Ponting and Aravind, 1999). In lipoid CAH, although the enzymes necessary for cholesterol metabolism are present, mutated cells are incapable of transferring cholesterol into mitochondria. StAR is involved in congenital lipoidic hyperplasia; mutations inactivating the protein in three unrelated individuals have been found to be associated with the disease (Lin *et al.*, 1995). Disruption of the functional StAR gene in transgenic mice has confirmed this view (Caron *et al.*, 1997). *In vitro* expression, localization of functional StAR and its mutant derivatives have shown that it is an outer mitochondrial membrane protein involved in the transfer of cholesterol to the inner mitochondrial membrane (Arakane *et al.*, 1998a,b; Wang *et al.*, 1998). Structural work on the START domain from another member of the family, namely MNL64, has indicated that these proteins could accommodate cholesterol molecules and shuttle them to the inner mitochondria (Tsujishita and Hurley, 2000). An interesting question that remains, however, is precisely how and why StAR is so efficient that it vastly increases cholesterol metabolism either *in vitro* or *in vivo*, as a mitochondrial protein or as a cytosolic protein (this has been reviewed in Tsujishita and Hurley, 2000). It is also worth pointing out that only a few of the proteins involved in steroid movement inside the cells have been characterized.

The mitochondria steps: transformation of cholesterol into pregnenolone and transformation of 11-deoxycortisol into hydrocortisone

ADR, ADX, AND MITOCHONDRIAL P450S

After a complex journey where NPC1, 2, StAR and probably other proteins play a fundamental role, cholesterol finally reaches the inner mitochondrial membrane,

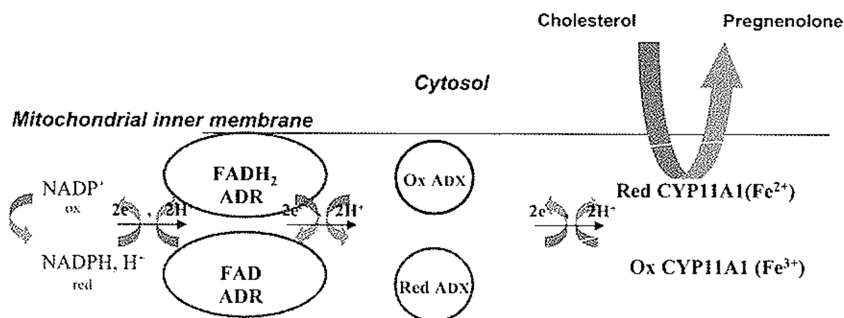


Figure 11.3. Scheme of electron transfer involved in mitochondrial P450 activities (CYP11A1 taken as an example). Ox = oxidized, Red = reduced, ADX = adrenodoxin, ADR = adrenodoxin reductase.

where it is readily metabolized into pregenolone (*Figure 11.2*). This reaction is performed by a typical mammalian mitochondrial P450 CYP11A1, which receives electrons for the splitting of molecular oxygen from NADPH through a specific transport chain composed of adrenodoxin reductase (ADR) and adrenodoxin (ADX) (*Figure 11.3*). The membrane-bound flavoprotein ADR transfers electrons from NADPH to its FAD moiety. ADX, a small soluble iron–sulfur protein (Lambeth and Pember, 1983), shuttles one electron at a time from reduced ADR (Gnanaiah and Omdahl, 1986) to CYP11A1 or other cytochrome P450 localized into mitochondria, such as P45011β1 (CYP11B1) (Lambeth *et al.*, 1979). We will now review the mitochondrial step of the pathway in the order of the electron movement from NADPH to the substrate via ADR, ADX, and P450s.

The conversion activity of these mitochondrial P450s has been investigated mostly by HPLC or GC analysis of the products after incubation with the solubilized substrates and the two electron carriers in the presence of NADPH. ADR activity can be analysed by taking advantage of its capacity to reduce ferricyanide, while ADX can be analysed in the presence of ADR by its capacity to reduce cytochrome *c*, both in the presence of NADPH. Physical methods have also been used for ADX, such as EPR and UV spectral analysis, redox potential measurement, and circular dichroism. For the CYP11A1/CYP11B1 system, techniques such as CO difference spectral analysis and substrate binding difference spectral analysis are used. For CYP11A1 or CYP11B1 in the presence of ADX and ADR, increase at 450 nm of the ferrous carbon monoxide complex can be monitored as the P450 heme moiety gains one electron.

In order to study the dynamics of the interactions between ADR, ADX, and their cytochrome P450 partners, purified proteins were first chemically modified with reagents such as diethyl pyrocarbonate, pyridoxal phosphate, and tetranitromethane, targeting specific amino acids of the respective proteins. The kinetics parameters of the modified proteins were then analysed. Cloning of the cDNAs permitted the expression and production of the natural mature proteins (bearing an extra initiator methionine). Single point mutations corresponding to important residues of the quoted proteins were introduced in their cDNAs, and the mutated proteins were

produced. The activity of the modified protein was assessed, refining the findings obtained with chemical reagents using the techniques described above.

ADR

ADR is a loosely membrane-associated flavoprotein of the mitochondrial matrix, which is synthesized as a precursor. Upon entering mitochondria, the pre-sequence is cleaved off to form the mature protein. ADR receives two electrons from NADPH and 'stores' them on its $FADH_2$ moiety. One electron is then delivered to its partner, ADX.

ADR purified from bovine adrenals showed an apparent molecular weight of 54 kDa and contains one mole of flavin moiety per mole of ADR (Chu and Kimura, 1973). At a pH 7.0, the redox potential was measured at –274 mVolts, K_m was measured at 1.82 μM for NADPH, and 5.56 mM for NADH. Lambeth and Kamin (1976) have confirmed the inhibitory effect of $NADP^+$ and showed the existence of a two-electron complex containing ADR, $FADH_2$, and $NADP^+$. The reduction of ADR by NADPH has been shown to be a first order reaction with a k_{app} value of 28 s^{-1} when using equimolar concentrations of ADR and NADPH (Lambeth and Kamin, 1976). Moreover, interaction between ADX and ADR has been shown to be influenced by ionic strength (Lambeth and Kamin, 1976; Vickery, 1997; Schiffler *et al.*, 2004b). Functional ADR from human or bovine origin can be expressed in *E. coli* (Brandt and Vickery, 1992; Sagara *et al.*, 1993) or *S. cerevisiae* (Akiyoshi-Shibata *et al.*, 1991) as a mature form devoid of its natural targeting sequence but including an N-terminal methionine. Interestingly, mature ADR, when targeted to the *S. cerevisiae* mitochondria, was found inactive for an as yet unknown reason (Akiyoshi-Shibata *et al.*, 1991; Dumas *et al.*, 1996). Unexpectedly, a natural *S. cerevisiae* protein, Arh1p (ADR related homologue 1), can advantageously replace mammalian mitochondrial ADR in transferring electrons to ADX and form a functional complex with CYP11B1 (Dumas *et al.*, 1996) with almost identical characteristics to the *bona fide* mammalian ADR, with the exception that it could use indifferently NADH or NADPH for its function *in vitro* (Lacour *et al.*, 1998). Such a protein was found to be an essential molecule of yeast (Lacour *et al.*, 1998; Manzella *et al.*, 1998) and has been shown to be involved in iron homeostasis (Lange *et al.*, 2000; Li *et al.*, 2001) and heme biosynthesis through a yeast mitochondrial ferredoxin homologous to ADX (Yah1p) (Barros *et al.*, 2002). An ADX/ADR equivalent has also been reported to exist in fission yeast (Bureik *et al.*, 2002b). Since the existence of an ADR homologue in yeast appears to be linked to the absence of mitochondrial P450 in yeast, it is possible that ADR too has additional functions in mammals related to iron metabolism. Compared to other partners of the steroid mitochondrial synthesis, ADR has been poorly studied. Using purified bovine natural ADX and ADR, residue Glu^4 of ADR could be cross-linked to residue Lys^{66} of ADX (Vickery, 1997). Mutagenesis experiments on Arg^{240} and Arg^{244} of recombinant purified human ADR showed that these two residues were important for binding of ADX on to ADR, as measured with a cytochrome *c* reduction assay (Vickery, 1997). This study, together with mutagenesis performed on ADX cDNAs (residues Asp^{76} and Asp^{79}, see below), implies that the interaction mediating electron transfer was electrostatic in nature. A bovine mature ADR was crystallized either as a complex with ADX (Lapko *et al.*,

1997; Muller *et al.*, 2001) or as a free form (Vonrhein *et al.*, 1999; Ziegler *et al.*, 1999), demonstrating an asymmetric charge distribution; a feature that has provided some clue about the mechanism of interaction with ADX. Docking of the ADX structure into the ADR has confirmed the importance of residues Glu^4, Arg^{240}, and Arg^{244} as the region of interaction between ADX and ADR. Recently, using rapid mixing techniques, an optical biosensor system, and recombinant ADX and ADR from bovine origin, the apparent constant rate of ADR reduction by NADPH under the same conditions as above has been measured at 17.4 s^{-1}, together with a K_d of 85 nM (Schiffler *et al.*, 2004b). This finding is in good agreement with the previous data of Lambeth and Kamin (1976).

ADX

In contrast to ADR, ADX is a 14.4 kDa soluble iron polypeptide, which contains [2Fe–2S] iron clusters as a redox active group. Natural ADX was purified from sources such as placenta or adrenals from human and bovine origin (Lambeth and Kamin, 1976). Mature ADX (where the natural N-terminal amphiphilic mitochondrial pre-sequence was replaced by an initiator methionine) was expressed as a functional polypeptide in *E. coli* (Coghlan and Vickery, 1989; Palin *et al.*, 1992; Sagara *et al.*, 1992; Uhlmann *et al.*, 1992; Bera and Bernhardt, 1999) and *S. cerevisiae* (Akiyoshi-Shibata *et al.*, 1991; Duport *et al.*, 1998). Using a yeast mitochondrial targeting sequence, a functional ADX could be directed to the *S. cerevisiae* mitochondrial matrix (Akiyoshi-Shibata *et al.*, 1991; Dumas *et al.*, 1996). This yeast-made ADX properly transfers electrons to recombinant CYP11B1 localized at the mitochondrial matrix (Cauet *et al.*, 2001). In order to study the different functions of this [2Fe–2S] protein (i.e. iron–sulfur cluster assembly, interactions with ADR, interactions with CYP11A1 or CYP11B1, electron transfer capacity), more than 30 mutants were designed and studied: this research has been elegantly reviewed by Grinberg and co-workers (Grinberg *et al.*, 2000). Crystallization of both the full-length mature ADX (1–128) (Pikuleva *et al.*, 2000) and of the short form of ADX (4–108, short ADX) (Muller *et al.*, 1998) has permitted the corroboration of the results obtained from comparing WT and mutant ADX activities. The four cysteines involved in the tetrahedral coordination of the two iron molecules have been identified to be the residues 46, 52, 55, and 92 (Uhlmann *et al.*, 1992). Interactions between ADX and ADR, ADX and P450 have been shown to be of electrostatic nature (Vickery, 1997). Finally, key ADX residues for both interactions (conserved in mammalian ADX) with ADR and CYP11A1 were negatively charged residues, such as Asp^{72}, Glu^{73}, Asp^{76}, and Asp^{79} (Vickery, 1997). Mutation of His^{56} residue was first recognized to increase the K_m of ADX in a cytochrome *c* reduction assay from 2 to 5 times, depending on the mutation introduced without changing the V_{max} (Beckert *et al.*, 1995). Subsequent studies focused on the relative binding affinity of ADX to CYP11A1, together with the steady state parameters for steroid hydroxylation. The K_m of ADX was shown to increase to more than 6 times without significant modification of the V_{max} (Beckert and Bernhardt, 1997). This effect was more pronounced for CYP11B1 than CYP11A1 (Grinberg *et al.*, 2000). Mutation of Tyr^{82} showed little difference in the kinetics of electron transfer between ADX and ADR (Beckert *et al.*, 1994). However, a Tyr^{82}Phe replace-

ment increases the affinity of ADX for CYP11A1, while Tyr^{82}Ser and Tyr^{82}Leu decrease the affinity (Beckert *et al.*, 1994). These studies confirmed the finding derived from chemical modifications of ADX. Overall, it can be said that the plasticity of ADX is remarkable, since there are only a few mutations, namely on Asp^{76} and Asp^{79} of human ADX, that are totally detrimental to the activity. Most changes have small, but still detectable, effects on the various kinetic parameters involving ADX. By contrast to ADX, only a few mutants have been described to map the interaction region of ADR and CYP11A1 with ADX.

Two residues fundamental for the ADX activity, i.e. Thr^{54} and Pro^{108}, were deciphered. Thr^{54}, a residue in the vicinity of the iron–sulfur cluster, is directly responsible for stabilization of the cluster (Uhlmann and Bernhardt, 1995). Pro^{108}, which is often conserved among the bacterial, fungal, and vertebrate ferredoxin, is crucial for the proper folding of ADX through hydrophobic contacts with Ile^{58}, His^{56} and Tyr^{82}, and hydrogen bonding with Arg^{14} (Grinberg and Bernhardt, 1998a,b). Interestingly, residues 1 to 3 and 109 to 124 could be trimmed off the full length without a significant change of the K_d of ADX for ADR (17 nM; Sagara *et al.*, 1993) to give rise to the important short ADX (4–108). By contrast, the V_{max} value was shown to increase by a factor of 1.7 (Uhlmann *et al.*, 1994). Further change of the Arg^{14} into an Ala improved the V_{max} value by a factor of 2.8 on the basis of cytochrome *c* reduction (Grinberg and Bernhardt, 1998a). As an example, the redox potential of mature ADX was modified from –273 mV for WT ADX down to –344 mV for short ADX. Unexpectedly, this short ADX was shown to improve the V_{max} of CYP11B1 by more than 3-fold in the 11β hydroxylation of corticosterone with a reduced K_m, whilst no effect was observed with CYP11A1 (Uhlmann *et al.*, 1994). Measuring the efficacy of this short ADX molecule to reduce CYP11B1 revealed that the apparent k_{cat} was improved by more than 4-fold (Uhlmann *et al.*, 1994). In other words, it seems that by increasing the affinity of ADX for its P450 partner, one can increase in the same fashion the velocity of electron transfer between the two proteins. To further elucidate this mechanism, Bernhardt and co-workers designed a new set of ADX mutants based on the idea that to obtain a very efficient ADX you have to combine a low K_d between ADX and its P450 partner together with a high electron transfer capacity (Schiffler *et al.*, 2001). The equivalent of ADX in the bacterial world – namely putidaredoxin – is very efficient for transferring electrons to the high turnover number bacterial P450s, such as CYP101 (P450cam) and CYP108 (P450terp) (Purdy *et al.*, 2004). In putidaredoxin, tryptophan in position 106 has been shown to be of primary importance for binding and electron transfer to CYP101 (Davies and Sligar, 1992). Alignment of putidaredoxin and bovine adrenodoxin has shown that the equivalent of Trp^{106} was Ser^{112} (Grinberg *et al.*, 2000). Knowing that adrenodoxin and putidaredoxin cannot substitute for one another in their respective reactions, researchers decided to construct a few mutants including the Tyr^{82}Phe/Ser^{112}Trp mutant (FW mutant), knowing that this particular Tyr^{82}Phe change was giving ADX a better affinity for ADR (Beckert *et al.*, 1994). Interestingly, the FW mutation not only increased the apparent rate of first electron transfer to CYP11A1 but also improved the capacity of CYP11A1 to convert cholesterol into pregnenolone, increasing the k_{cat} from 0.6 min^{-1} to 63 min^{-1} and decreasing the K_m from 3.24 μM down to 0.33 μM (Schiffler *et al.*, 2001). Moreover, mutant FW was also shown to have a better affinity for CYP11A1 than the WT

adrenodoxin. Surprisingly, mutant FW showed no better performance than the WT adrenodoxin in CYP11B1 assays (Schiffler *et al.*, 2001). It is not known whether this improvement has any biological significance and if it can be achieved *in vivo* in the inner mitochondrial membrane. Nonetheless, this research has clearly demonstrated the impressive plasticity of ADX and the key importance of electron transfer for CYP11A1 and CYP11B1 activities.

CYP11A1, CYP11B1

The final destination of the electrons acquired by ADX is a small family of cytochrome P450s from the mitochondrial matrix, and these include CYP11A1 and CYP11B1. These two mitochondrial P450 hydroxylases perform key reactions for the synthesis of steroids in mammals. They are localized in specialized tissues of the gonads, placenta, and adrenal cortex (Ishimura and Fujita, 1997). The presence of CYP11A1 has also been reported in specific parts of the brain (Baulieu *et al.*, 2001). CYP11A1 transforms cholesterol into pregnenolone (*Figure 11.2*), while CYP11B1 has, among other hydroxylating activities, the capacity to transform 11-deoxycortisol into hydrocortisone (Yanagibashi *et al.*, 1986). In some species, such as *Bos taurus*, a single polypeptide contains the two hydroxylase activities (11β-hydroxylase and 18-hydroxylase) and the aldosterone synthase activity (Wada *et al.*, 1985; Delorme *et al.*, 1995); in other species, such as *Homo sapiens*, one polypeptide, CYP11B1, has mostly an 11β-hydroxylase activity, while the second polypeptide, CYP11B2, has mostly the aldosterone synthase activity (Bureik *et al.*, 2002b). Recently, the molecular biology of these two mitochondrial P450 hydroxylases has been reviewed extensively (Bureik *et al.*, 2002a; Lisurek and Bernhardt, 2004). Although the involvement of these polypeptides in steroidogenesis can be considered as their main function, CYP11A1 has been shown to be able to transform 1,25-hydroxyvitamin D_3 and 7-dehydrocholesterol into 20-hydroxyvitamin D_3, 20,22-dihydroxyvitamin D_3 and 7-dehydropregnenolone, respectively (Guryev *et al.*, 2003), while a possible capacity of transforming xenobiotics has been reported for CYP11B1 (Lund and Lund, 1995). In these studies, CYP11A1 and CYP11B1 had been purified from bovine adrenals (Shikita and Hall, 1973; Katagiri *et al.*, 1976; Wada *et al.*, 1985), whereas human CYP11A1 had been purified from placenta (Tuckey and Cameron, 1993b). Both CYP11A1 and CYP11B1 have a small turnover number. A k_{cat} for cholesterol of 12 min^{-1} and 34 min^{-1} was described corresponding to a K_m of 77 μM and 70 μM, respectively, for bovine and human CYP11A1 (Tuckey and Cameron, 1993b). For bovine CYP11B1, a k_{cat} of 40 min^{-1} for transformation of deoxycorticosterone into corticosterone with a K_m of 1.5 μM has been described (Wada *et al.*, 1988; Delorme *et al.*, 1995). In our experiments in our own laboratory, using purified bovine CYP11B1 for transformation of 11-deoxycortisol into hydrocortisone, a k_{cat} of 60 min^{-1} has been measured with a K_m of 5 μM, the same catalytic efficiency was found for 11-deoxycorticosterone (Cauet, unpublished results).

It is believed that the side-chain of cholesterol is cleaved off by a mechanism involving two successive hydroxylations at position 20, 22, and a final cleavage of the resulting glycol to give pregnenolone and isocaproic acid (this has been reviewed in Lieberman and Lin, 2001). Whether the hydroxylated cholesterols are true intermediates or not is a matter of controversy. Some of the intermediates

have been isolated from mammalian mitochondria (Teicher *et al.*, 1978; Tuckey and Cameron, 1993a; Sugano *et al.*, 1996). Moreover, it appears that 22R-hydroxy cholesterol and 22R-20α-dihydroxy cholesterol are *in vitro* better substrates than cholesterol using purified preparations of bovine CYP11A1 (Morisaki *et al.*, 1980; Tuckey and Cameron, 1993b). 22R-hydroxy cholesterol might play a role in the regulation of the pathway since it is a potent agonist of the nuclear receptor, LXR, which has been implicated in cholesterol homeostasis (Lehmann *et al.*, 1997; Venkateswaran *et al.*, 2000). Using CYP11A1 purified from human placenta or from bovine adrenals, it has been possible to study CYP11A1 side-chain cleaving specificity. An important set of different side-chains with two or more hydroxyls, different positions of the double bond, different lengths and branching of the side-chains, was studied (Morisaki *et al.*, 1980; Tuckey and Cameron, 1993b). Only a few derivatives of cholesterol could not be metabolized by CYP11A1, i.e. side-chains with 9 carbons or more, side-chains with two hydroxyls at position 23 and 24, side-chains with a double bond at position 22, 23, and finally side-chains with only 2 methyl groups. Elongation of the side-chain at position 24 showed a decreased K_m together with a decreased k_{cat}, while in the possible combination of hydroxylation at position 20 and 22, only 22R-OH-cholesterol and 22R-20αOH-cholesterol showed a decreased K_m (about 10 times lower), together with an improved k_{cat} up to 2.5 and 5.5 times for the human enzyme (Tuckey and Cameron, 1993b), respectively. Hydroxylations at positions 24, 25, and 26 were detrimental for the side-chain cleaving activity down to a few per cent of their original value (Tuckey and Cameron, 1993b). Esterification at position 3 was also assessed; only cholesterol sulfate could be metabolized with the same catalytic efficiency but with a much higher K_m (297 μM) than cholesterol (70 μM), whereas all the other esters (methyl ether, formate, acetate, propionate, butyrate, hexanoate, caprylate) showed a reduced k_{cat} (Tuckey *et al.*, 1996). Unlike CYP11B1, CYP11A1 and *in vitro* conceived mutants were expressed in *E. coli* as a mature polypeptide (Wada *et al.*, 1991; Wada and Waterman, 1992; Woods *et al.*, 1998; Usanov *et al.*, 2002; Pikuleva, 2004) and in *S. cerevisiae* as a mature polypeptide (Duport *et al.*, 1998) or targeted to mitochondria (Cauet *et al.*, 2001). The kinetic parameters of CYP11A1 were only measured on the *E. coli*-produced enzyme, so it was not possible to assess for the quality of the *S. cerevisiae*-produced polypeptide. k_{cat} measured on bovine CYP11A1 either purified from bovine adrenals or produced in *E. coli* were in the same range, 14 min^{-1} (in a buffer containing 1 μM ADX) (Wada and Waterman, 1992). For the human form, both k_{cat} and K_m were reported to be in the same range, i.e. 15 to 25 min^{-1}, with a K_m for cholesterol ranging from 70 to 150 μM (Woods *et al.*, 1998). These findings permitted the design and production of mutants at position 339 and 343, which correspond to a pair of conserved positively charged lysine residues among the mitochondrial P450s (Wada and Waterman, 1992). Mutations of these two residues independently into glutamine or glutamic acid very efficiently decrease the capacity of ADX to bind to CYP11A1 (Wada and Waterman, 1992). Moreover, the double glutamate mutant (Lys^{339}Gln, Lys^{343}Glu) had almost no affinity for CYP11A1 (Wada and Waterman, 1992). More recently, an effort has been made to corroborate results obtained from protein chemistry on CYP11A1 Lys residues (103, 194, 109, 110, 145, 148, 394, 403, 405) and Arg residues drawn from comparison in the mitochondrial P450 family and, pointing out their conservation

at positions 425 and 426, an expression of mutated CYP11A1 was started (Usanov *et al.*, 2002). Combining molecular modelling and *in vitro* mutagenesis, a CYP11A1 molecular model was constructed using P450BMP docked with the published ADX structure (Usanov *et al.*, 2002). Mutants bearing individually replaced Lys residues (103, 110, 145, 394, 403, 405) or Arg residue (426) by Gln could be produced and purified from *E. coli* (Usanov *et al.*, 2002). Interestingly, all the purified CYP11A1 mutants (except for those mutated at positions 110 and 145) demonstrated a reduced capacity to bind ADX together with a typical CO difference spectrum, indicating (together with other spectral properties) that no serious structural changes had been introduced in these proteins. The most seriously affected mutants were the $Lys^{405}Gln$ and $Arg^{426}Gln$. The latter had no measurable affinity for ADX, the former showed a lower capacity to bind ADX up to 1.1 μM from 0.15 μM (Usanov *et al.*, 2002). Using the molecular model, it was possible to decipher some of the residues implicated in the ADX–CYP11A1 interaction. Four salt bridges could be identified: Lys^{403}, Lys^{405}, Arg^{426}, and Lys^{267} from CYP11A1 towards Asp^{76}, Asp^{72}, Glu^{73}, and Glu^{47} of ADX, respectively. In other words, electrostatic interaction is mainly responsible for the binding of ADX on to ADR. In *S. cerevisiae*, for bovine CYP11A1 two approaches have been tried, either to target it inside the mitochondria or as maturated polypeptide bearing an extra methionine. CYP11A1 was either targeted inside the mitochondria using classical targeting pre-sequences (Savelev *et al.*, 1997; Cauet *et al.*, 2001) or expressed as a mature polypeptide (Duport *et al.*, 1998). The mitochondrial targeted polypeptide was detected in the inner mitochondrial membrane of yeast (Cauet *et al.*, 2001), while, unexpectedly, the mature form was detected mostly at the plasma membrane (Duport *et al.*, 2003). *In vitro* mitochondrial CYP11A1 reconstituted activity could be recovered from mitochondria after addition of purified ADX, ADR, and substrate (Savelev *et al.*, 1997; Cauet *et al.*, 2001). Crude membrane preparations of yeast expressing mature CYP11A1 facilitated the reconstitution under the same conditions of a pregnenolone-producing activity (Duport and Pompon, unpublished results). *In vivo*, when mature forms of CYP11A1, ADX, and ADR were expressed in the same strain producing the suitable substrate, pregnenolone could be detected in the medium (Duport *et al.*, 1998). In contrast, when the CYP11A1 was targeted to mitochondria, together with ADX and the endogenous ARH1p, very little *in vivo* activity could be recovered (unpublished results) (Cauet *et al.*, 2001). It is not known whether this phenomenon is due to the absence of mitochondrial sterol substrate or the absence of accessible functional CYP11A1. It confirms the difficulty of targeting a functional CYP11A1 to the mitochondria of a recombinant host, and is consistent with the observations of others (Takagi *et al.*, 1992; Savelev *et al.*, 1997). In the absence of spectral and kinetics studies, it is not possible to evaluate the quality of the produced polypeptides.

In contrast with other cytochrome P450s, CYP11B1 and CYP11B2 are difficult to express in *E. coli*. Limited success has been obtained with rat CYP11B1 and CYP11B2, which have been expressed at 0.1% of the total cellular protein (Nonaka *et al.*, 1998). In that work, a k_{cat} of about 110 min^{-1} for CYP11B1 and corticosterone was reported and compared with the 22 min^{-1} of the native rat enzyme (Nonaka *et al.*, 1998). Bovine CYP11B1, and both human CYP11B1 and CYP11B2, could only be expressed in yeast – either *S. pombe* or *S. cerevisiae* (Dumas *et al.*, 1996; Bureik *et*

al., 2002b; Szczebara *et al.*, 2003). *In vivo*, recombinant *S. cerevisiae* expressing bovine CYP11A1 and ADX, both targeted to their mitochondria, was able to transform 11-deoxycortisol into hydrocortisone with a small efficacy producing up to 47 nmoles l^{-1} (Dumas *et al.*, 1996). *In vitro*, using radiolabelled 11-deoxycortisol, a small activity was recovered in the mitochondrial fraction of yeast expressing the bovine enzyme (Dumas *et al.*, 1996). This activity was later traced down to the inner mitochondrial membrane of the recombinant yeast (Cauet *et al.*, 2001). No *in vitro* data was published concerning the recombinant human CYP11B1 and CYP11B2. Quite impressively, CYP11B2 has been shown by Bureik and colleagues to function in *S. pombe in vivo* with its natural pre-sequence and together with electron carriers provided by the host (Bureik *et al.*, 2002b). A production of 700 nmoles l^{-1} and per day was achieved (Bureik *et al.*, 2002b). The quality of the human CYP11B2 polypeptides produced in yeast was not evaluated (Bureik *et al.*, 2002b). These generally poor expression levels impair the possibility to study in depth the interaction between human and bovine CYP11B1 and its partners. One must therefore rely on *in vivo* experiments, although these are difficult to interpret.

THE STOICHIOMETRY AND EQUILIBRIA FOR MAXIMUM CONVERSION USING CYP11A1, CYP11B1, ADX, AND ADR

It is difficult to infer how well the reconstituted CYP11A1 or CYP11B1 system is representative of the *in vivo* situation of the mitochondrial membrane, i.e. if the adrenal cholesterol present in mitochondria is in a situation where it can be metabolized readily by CYP11A1 into pregnenolone. Thus, the first level of regulation comes from the availability and solubility of the susbtrate. *In vitro*, the notoriously insoluble cholesterol is rendered soluble using a detergent such as Tween 20. Addition of Tween 20 clearly increases the catalytic activity of CYP11A1, probably through an increased solubility of the substrate (Tuckey *et al.*, 2001). Lipids, such as mitochondrial cardiolipin, play a second role, such as enhancing the maximum velocity of pregnenolone formation (Kowluru *et al.*, 1983; Kisselev *et al.*, 1999). The same observation has been made with the aldosterone synthase catalytic activity of bovine CYP11B1 that was enhanced by addition of mitochondrial lipidic extracts (Wada *et al.*, 1985). Thus, mitochondrial lipids of the inner membrane clearly play a role in regulating steroid biosynthesis at the level of the side-chain cleavage, aldosterone synthase, and probably 11β-hydroxylase activities (Imai *et al.*, 1998).

Electron flux is a second point of regulation obtained by the balancing of ADX and ADR expression at constant P450 expression level. In an effort to design a simple expression system where ADX, ADR, and cytochrome P450 are in stoichiometric amounts, fusions between CYP11A1 or CYP11B1, ADX, and ADR were reported (Harikrishna *et al.*, 1993; Miller, 1995; Cao *et al.*, 2000; Novikova *et al.*, 2000; Huang and Miller, 2001). These fusions, although functioning *in vivo*, have less activity than the *bona fide* three-component system. Moreover, addition of an excess of reducing equivalents increases the activity recovered from the triple fusion, indicating that the electron transfer is limited in the 3-protein system (Cao *et al.*, 2000). This observation is not unexpected, considering the complexity of the electron transfer between ADR, ADX, and mitochondrial P450s.

In mammalian cells naturally producing steroids, the situation is diverse. A differential level of expression for ADX and ADR was observed for tissue producing steroids *in vivo*. In bovine corpus luteum and adrenal cortex ADR is in excess, so that ADX is readily reduced (Hanukoglu and Hanukoglu, 1986), while in human placenta ADR is limiting, so that oxidized ADX is acting as a competitive inhibitor of CYP11A1 (Tuckey *et al.*, 2001). CYP11A1 and CYP11B1 are in excess in the corpus luteum and adrenal cortex (Hanukoglu and Hanukoglu, 1986), and probably also in the placenta. *In vitro*, in a reconstituted system, the molar ratio of ADR/ADX/CYP11A1 can be modulated. In general, activities are measured in conditions where CYP11A1 concentration is limiting, varying from 50 nM to 1 μM, but not the electron transfer capacity. It is interesting to compare molar ratio of ADR/ADX/CYP11A1 (M/M/M) of *in vitro* reconstituted CYP11A1 assay used by different authors: 4/16/1 (Pikuleva, 2004); 0.5/2/1 (Usanov *et al.*, 2002); 1/1–128/1 (Schiffler *et al.*, 2001); 5/100/1 (Tuckey *et al.*, 1996); 5/5–500/1 (Wada and Waterman, 1992). The latter researchers observed that increasing the adrenodoxin concentration 100-fold has a minor but detectable change of CYP11A1 k_{cat} passing from 13.8 to 19.5 min^{-1}. The three-component system is working at its maximum when ADR is in slight excess over CYP11A1, while ADX is in vast excess over its two partners. Recently, another factor, i.e. ionic strength, has been shown to be capable of decreasing the catalytic efficiency (k_{cat}/K_m) of CYP11A1 by a factor of 35.7. In order to reconstitute *in vivo* a viable and efficient CYP11A1 system, a balance between substrate availability, electron transfer capacity, ionic strength, and CYP11A1 level has to be reached. In mammalian steroid-producing cells, fine tuning of pathway flux can be achieved by choosing the appropriate NADPH concentration, substrate availability, the balance between ADX and ADR, CYP11A1 expression, and the ionic strength. In a recombinant system, it remains to be seen how the flux of electrons can affect the overall specificity and capacity of P450 reaction. However, in mammalian transfection experiments using bovine CYP11B1, an excess of reducing equivalent stimulates its 11β-hydroxylating capacity versus its aldosterone synthase activity (Cao and Bernhardt, 1999).

INTERACTION BETWEEN CYP11A1, CYP11B1, AND CYP11B2

In 1992, it was first recognized that bovine CYP11A1 could improve the bovine CYP11B1 catalytic activity towards 11-deoxycortocisterone in a dose-dependent fashion.

This allosteric interaction is now thought to take place in the bovine adrenal mitochondria in order to shut off the aldosterone activity of the bovine CYP11B1 (Kominami *et al.*, 1994; Imai *et al.*, 1998). This phenomenon was used in a biotechnological approach in order to improve the 11β-hydroxylase activity of bovine CYP11B1 activity towards 11-deoxycortisol (Cauet *et al.*, 2001). *In vivo* and in *S. cerevisiae*, this activity could be improved by a factor of 3 using CYP11A1 and CYP11B1 expressing cells. This improvement was probably due to direct interaction between CYP11A1 and CYP11B1 in the inner mitochondrial membrane (Cauet *et al.*, 2001). This stimulation is only observed when there is an excess of reduced ADX, as there is an apparent competition for reducing equivalent between CYP11A1 and CYP11B1 in the favour of CYP11B1, in rat mitochondria and in

transfected mammalian cells (Yamazaki *et al.*, 1993; Cao and Bernhardt, 1999). This stimulation is apparently species specific, since it is not observed with human CYP11A1 and CYP11B1, even in the presence of saturating amounts of reducing equivalents. The biological significance and mechanism of this stimulation remains to be studied.

MECHANISM OF ACTION

Earlier reports mentioned the existence of a high molecular weight complex containing the steroidogenic enzymes in specialized cells (Shikita and Hall, 1973). Since then, four working models for transfer of reducing equivalent from NADPH on to P450 have been proposed: shuttle model; shuttle model with ADX dimers; the 1ADR:1 oxidized ADX, 1 reduced ADX:1P450; the 1ADR:1 oxidized ADX:1P450 (Beilke *et al.*, 2002). The difficulty of designing a reliable model resides on the facts that ADX has the same contact sites with ADR and mitochondrial P450 (see above), and that ADX is probably acting as a dimer (Pikuleva *et al.*, 2000). So, a complex containing the three proteins is rather unlikely. Our own experience in yeast has shown that a functional electron transfer takes place with mature ADR in the endoplasmatic reticulum, soluble ADX, and a plasma membrane localized CYP11A1 (Duport *et al.*, 2003). Recent reports using techniques as diverse as NMR spectroscopy, crystallography, surface plasmon resonance, and stopped flow analysis have shed a new light on this specialized electron transfer (Muller *et al.*, 2001; Beilke *et al.*, 2002; Schiffler *et al.*, 2004b). In the cascade of three reduction events comprising reduction of ADR, then ADX, and then P450, ADR is the fastest to be reduced with an apparent rate constant of 32 s^{-1}, while ADX reduction has a rate constant of 5.4 s^{-1}. Finally, CYP11A1 reduction with the first electron has a slow rate constant of 1.9 s^{-1}, and the second electron transfer has an even slower rate constant of 0.004 s^{-1}. These observations corroborate previous results, which indicated that one way to improve CYP11A1 is to use an ADX mutant with a greater capacity to transfer electrons.

Upon reduction of ADR, by NADPH, the electrons are transferred to the flavin moiety of ADR. Crystallization of the ADX/ADR complex allowed precise identification of the contacts between the two molecules and showed that the $FADH_2$ moiety was within a 10 Å distance of the iron–sulfur moiety of ADX (Muller *et al.*, 2001). This distance should permit electron transfer between the two molecules. Interestingly, the k_{on} rates for ADX to ADR and for ADX to CYP11A1 have been measured as 4400 and 691 000, respectively, with a K_d of 90 and 13 nM as oxidized proteins, while the k_{off} rates were shown to be very low in a study by Schiffler and co-workers (Schiffler *et al.*, 2004b). It is apparent that the complex between reduced ADX and CYP11A1 is very stable, while there are conflicting reports about the stability of the ADX/ADR complex. Interestingly, these measurements are very much influenced by the ionic strength of the medium; there is apparently an optimal salt concentration for obtaining a maximal CYP11A1 activity (Schiffler *et al.*, 2004b). It is difficult to infer how well these reconstituted systems are representing the *in vivo* situation of the inner mitochondrial membrane, but these experiments have furnished a wealth of information regarding the kinetics of electron transfer and product formation with this three-partner complex.

The second step: transformation of pregnenolone into progesterone

The pregnenolone produced in the mitochondria is transferred to the smooth endoplasmatic reticulum, where it is converted by a bi-functional enzyme to a C_{21} gestagen compound, the progesterone (*Figure 11.2*). The so-called 3β-HSD/isomerase 42 kDa homodimer enzyme responsible for that reaction is a 3β-hydroxysteroid dehydrogenase (dehydrogenization of C3 of the ring A) and a $\Delta^5 \rightarrow \Delta^4$ isomerase (isomerization of the double bound from the B-ring to the A-ring). The 3β-HSD/isomerase is a member of the short-chain dehydrogenase/reductase (SDR) family of enzymes that presents a conserved motif in the catalytic domain. It has also been found in *Streptomyces* 3α,20β-HSD, *Drosophila* alcohol dehydrogenase, and *E. coli* UDP-galactose 4-epimerase (Jornvall *et al.*, 1995). In 1992, it was demonstrated that purified enzyme from 3β-HSD/isomerase bovine adrenal cortex exhibits both dehydrogenase and isomerase activities (Cherradi *et al.*, 1992).

Two distinct genes expressed in tissue-specific pattern encode the two human isoforms of the enzyme. The human type I gene is almost exclusively expressed in the placenta, endometrium, and peripheral tissues, including mammary glands, prostate, and skin, whereas the type II gene is predominantly expressed in the human adrenal gland, ovary, and testis (Lachance *et al.*, 1991); this work has been reviewed by Simard and co-workers (Simard *et al.*, 1996). Type I 3β-HSD/isomerase catalyses the conversion of 3β-hydroxy-5-ene-steroids (DHEA, pregnenolone) to 3-oxo-4-ene-steroids (androstenedione, progesterone). Androstenedione is converted by placental aromatase and 17β-hydroxysteroid dehydrogenase (17β-HSD) to estradiol. In human adrenals, type II 3β-HSD/isomerase is required for the production of cortisone and aldosterone. The deficiency of the latter is responsible for a rare recessive autosomal form of congenital adrenal hyperplasia characterized by a severe impairment of steroid biosynthesis in both the adrenals and the gonads (Rheaume *et al.*, 1992). Type I and type II enzymes have 93.3% homology in their amino acid sequences. Studies with mutant forms of the enzyme show that His^{261} is linked to the 3β-HSD activities. Tyr^{154} and Lys^{158} are also essential residues of this catalytic domain. These studies also suggest that Tyr^{253} and Asp^{257} play a role in the isomerase activity. This region is part of the active site. Furthermore, the human isomerase activity requires allosteric activation by the coenzymes, NAD^+ and NADH (Mason *et al.*, 1998; Thomas *et al.*, 2004).

The human type I 3β-HSD/isomerase contains both enzyme activities (Thomas *et al.*, 2001). It resides in the endoplasmatic reticulum and mitochondria of human placental syncytiotrophoblast. In this peripheral tissue, it catalyses the conversion of dehydroepiandrosterone (DHEA) and pregnenolone to androstenedione and progesterone, respectively (Thomas *et al.*, 1989).

The 3β-HSD/isomerase is an enzyme mainly detected in the endoplasmatic reticulum. However, in bovine adrenals, this enzyme co-localizes with CYP11A1 in the inner parts and contact sites of mitochondrial membranes (Cherradi *et al.*, 1997). A third protein, namely StAR, capable of mobilizing cholesterol from the outer to the inner mitochondrial membrane (see above), was detected in the same fractions in a calcium-dependent fashion (Cherradi *et al.*, 1997). This observation suggests that CYP11A1, 3β-HSD, and StAR might associate into a molecular complex containing a functional steroidogenic unit. This set would facilitate the rapid transformation of

pregnenolone into progesterone (Cherradi *et al.*, 1995). This observation raises the possibility of a high molecular weight organization of CYP11A1, 3β-HSD/isomerase, StAR, and other proteins, although this has not yet been corroborated by data obtained for reconstituted material – at least for the moment.

Interestingly, 3β-HSD proteins have been selectively detected in human breast, colon, and cervix cancers. Indeed, the IL-4 and IL-13 cytokines cause a rapid and potent induction of 3β-HSD type I gene transcription in tumoral cell lines, as well as in normal tissues (Gingras *et al.*, 1999). Moreover, their biological effect is mediated through the Stat6 protein, a signal transducer and transcription activator. In fact, Stat6 binds two consensus sequences located in the 3β-HSD type I promoter (Gingras *et al.*, 1999).

In the particular case of oestrogen-induced breast cancer, IL-4 and IL-13 have an inhibitory effect on cell proliferation by repressing the expression of 3β-HSD type I gene. It is now well known that oestrogens stimulate cell growth of hormone-sensitive breast cancer cells, while androgens exert an antiproliferative effect (Poulin and Labrie, 1986; Poulin *et al.*, 1988). In other words, this 3β-HSD type I gene expression is relevant in physiological and pathological conditions of various tissues. 3β-HSD proteins are selectively detected in human breast, colon, and cervix cancers (Gingras and Simard, 1999). The selective inhibition of this enzyme could block the conversion circulating DHEA into oestradiol, an activator of cancer cell growth.

The cDNAs of human 3β-HSD have been cloned in a wide variety of species. Human type I and II 3β-HSD were expressed in *S. cerevisiae* under the control of the weak *CYC1* promoter (Degryse *et al.*, 1999). The activities of the yeast-made 3β-HSD were measured in cell-free extracts with pregnenolone as a substrate. K_m values of 20 and 230 nM have been observed, respectively, for yeast-made type I and type II 3β-HSD (Degryse *et al.*, 1999). Using pregnenolone as a substrate and 3β-HSD prepared from human placenta, Gibb measured an apparent K_m value of ~40 nM (Gibb, 1981). So, it is possible to express a functional 3β-HSD in yeast having characteristics of the natural protein (Degryse *et al.*, 1999). No other reports have been made on the recombinant expression of 3β-HSD.

MICROSOMAL STEPS: HYDROXYLATIONS AT POSITIONS 17 AND 21

The small family of mitochondrial P450s is dwarfed by a much larger family of microsomal P450s of approximately 50 members (Lewis, 2004), all having the same CPR partner. This ubiquitous reductase and its 17- and 21-hydroxylase partners have been studied in much the same way as have ADX, ADR, CYP17A1, and CYP21A1.

The third step: transformation of progesterone into 17OH-progesterone

Pregnenolone and progesterone are key precursors of sex hormones (androgens and oestrogens), gluco- and mineralocorticoids. Both can be converted by CYP17A1 to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, respectively (*Figure 11.2*). These 17-hydroxylated steroids can undergo a scission of the C-17,20-carbon bond, to yield the 19-carbon androgens: DHEA and androstenedione, respectively.

All of these reactions are mediated by a single enzyme, P450$_{17}\alpha$ (CYP17A1), which presents both 17α-hydroxylase and C-17,20-lyase activities (C17–20 bond cleavage reactions of C21-steroids). As a mammalian microsomal P450, CYP17A1 relies for its activity on electrons provided by an FMN/FAD protein, namely P450 reductase. It has been shown that the CYP17A1s of guinea pig, mouse, rat, and hamster mainly synthesize androstenedione from progesterone, named the Δ^4 pathway (progesterone→17αOH progesterone→androstenedione), whereas those of human, bovine, and sheep produce DHEA from pregnenolone, via a process named the Δ^5 pathway (pregnenolone→17αOH-pregnenolone→DHEA) (Gilep *et al.*, 2004). It was first thought these CYP17A1 activities were performed by separate enzymes, localized in membranes of the smooth endoplasmatic reticula of adrenal and gonadal glands. CYP17A1 activity regulates three pathways: at the one end, the DHEA and androstenedione pathway; at the other end, the aldosterone pathway. When CYP17A1 activity is absent, the products are C-21–17-deoxysteroids, such as aldosterone, the major mineralocorticoid produced.

When the 17α-hydroxylase activity of P450c17 is present, C21–17-hydroxysteroids, such as cortisol, the main representative of the glucocorticoid family, are produced. When the 17α-hydroxylase and the 17,20-lyase activities of CYP17A1 are both present, C-19 precursors of sex steroids are produced. In any case, pregnenolone, a product of CYP11A1, remains the precursor of all these hormones. Thus, the presence or absence of CYP17A1 directs this pregnenolone towards its final metabolic pathway.

CYP17A1 is the only enzyme known in which a physiological process differentially regulates these multiple activities. Genetic studies have shown that there was a single species of CYP17A1 mRNA, identical in the human adrenal and gonad because a single-copy gene encoded it, termed *CYP17* (Miller, 2002).

It is known that, besides the reactions indicated, cytochrome CYP17A1 has some extra activity including, for example, 16α-hydroxylation of progesterone (Swart *et al.*, 2002). All this makes cytochrome CYP17A1 an intensively studied enzyme. It has been purified from different steroidogenic organs (Nakajin *et al.*, 1984; Perrin *et al.*, 1991). This 54–57 kDa protein has been easily expressed in either *E. coli* (Barnes *et al.*, 1991) or *S. cerevisiae* (Sakaki *et al.*, 1989, 1991). The reported k_{cat} with saturating amounts of NADPH P450 reductase varies from 1 min^{-1} for the *E. coli* expressed material (Barnes *et al.*, 1991) to 7 min^{-1} for that from the natural pig protein (Nakajin *et al.*, 1984). CYP17A1s for different species show a high degree of homology with respect to amino acid sequence (40–98%). These differences can affect the activities that can preferentially turn towards Δ^4- (progesterone derivatives) or Δ^5- steroids (pregnenolone derivatives) (Gilep *et al.*, 2003). Chimeric hemeproteins were engineered to contain fragments of both guinea pig (Δ^4-type) and bovine (Δ^5-type) CYP17A1 polypeptides. It was possible to evaluate the domains responsible for Δ^4-type and Δ^5-type biosynthetic pathways in a three-dimensional model (Gilep *et al.*, 2004). Using *E. coli* expressed guinea pig CYP17A1 protein and then chemical modification, some of the residues involved in membrane contacts were mapped (Izumi *et al.*, 2003). Three Lys (59, 490, 492) and Arg (211, 212, 216) residues appear to be protected *in vitro* by proteoliposomes (Izumi *et al.*, 2003).

The cDNA coding for the bovine CYP17A1 was expressed in yeast under control of the *CYC1* promoter or *ADH1* promoter from a 2-micron-based shuttle plasmid

(high copy number) (Sakaki *et al.*, 1989; Degryse *et al.*, 1999). Both *in vitro* and *in vivo*, a 17α-hydroxylase activity could be recovered, which probably couples with the yeast NADPH P450 reductase (see above). No evidence for an *in vivo* 17,20-lyase activity could be obtained on 17α-hydroxyprogesterone (Sakaki *et al.*, 1989; Degryse *et al.*, 1999). In the presence of an excess of purified rat liver NADPH–cytochrome P450 reductase, K_m values of 0.2 μM and 1.4 μM for pregnenolone and progesterone, respectively, were measured *in vitro*. A low conversion of 17-OH pregnenolone into DHEA could be detected *in vitro*, indicating some 17,20-lyase activity (Degryse *et al.*, 1999).

The fourth step: transformation of 17OH-progesterone into 11-deoxycortisol

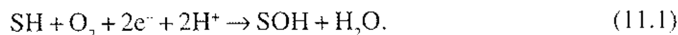
CYP17A1 and P450c21 (CYP21A1) are both ≈55 kDa microsomal cytochrome P450s that present hydroxylating activities, respectively, at positions C-17 and C-21 (*Figures 11.1* and *11. 2*). Both these activities are required for hydrocortisone biosynthesis. In contrast to CYP17A1, which has a greater range of substrates and activities, the only demonstrable activities for CYP21A1 have been the 21-hydroxylation of progesterone and 17α-hydroxyprogesterone to yield deoxy-corticosterone and 11-deoxycortisol, respectively (*Figure 11.2*). CYP17A1 and CYP21A1 polypeptides differ by only 14 amino acids in length, they share 29% amino acid identity and their genes present identical intron/exon organization. Since both polypeptides bind progesterone, we can infer that the two active sites must have common features.

The four different cytochrome P450s (CYP11A1, CYP17A1, CYP21A1, and CYP11B1), involved in hydrocortisone synthesis of the adrenal cortex can be affected by mutations, resulting in congenital adrenal hyperplasia (CAH). More than 90% of all cases of CAH that result in a decrease of plasmatic hydrocortisone and aldosterone are due to a deficiency in 21-hydroxylase activities. The complicated structure of the steroid 21-hydroxylase locus, with an active gene termed *CYP21* and a pseudogene *CYP21P*, predisposes to misalignment during meiosis, followed by recombination causing mutations, and thus a non-functional *CYP21* gene. The effects of mutations in the coding sequence of the *CYP21* gene have been studied by transfection of the CYP21A1 cDNAs in mammalian cells (Lajic *et al.*, 1999). Recombinant expression of CYP21A1 in *E. coli* was also used to decipher CYP21A1 CAH mutations. Chung and co-workers identified a Glu^{380}Asp mutation causing a reduced heme binding capacity of CYP21A1 (Hsu *et al.*, 1999). Finally, in a biotechnological approach, *S. cerevisiae* was used as a host for expression of CYP21A1. Using an alcohol dehydrogenase I promoter, CYP21A1 expressing cells were shown to be capable of metabolizing progesterone or 17α-hydroxyprogesterone with high efficiency *in vivo*. *In vitro*, the measured k_{cat} and K_m of recombinant CYP21A1 were, respectively, 28 min^{-1} and 0.29 μM for 17α–hydroxyprogesterone, in the same range as the value of 48 min^{-1} measured for the k_{cat} value of purified bovine CYP21A1.

NADPH P450 REDUCTASE AND CYTOCHROME *B5*

Cytochrome P450 proteins contain a single iron protoporphyrin IX prosthetic group. The heme iron is reduced from the ferric (Fe^{3+}) state to its ferrous (Fe^{2+}) state by

specific reductases. This process permits the binding of molecular oxygen to the heme iron moiety. For a typical hydroxylation reaction, two electrons are required. Molecular oxygen is split and one oxygen atom is incorporated into the substrate (S), while the other one is reduced into water according to:



$$SH + O_2 + 2e^- + 2H^+ \rightarrow SOH + H_2O. \quad (11.1)$$

As described earlier, mitochondrial P450s receive electrons from ADX: by contrast, microsomal P450s, including steroid hydroxylases, CYP17A1 and CYP21A1, receive electrons from a ubiquitous NADPH P450 oxidoreductase (CPR). This microsomal CPR protein, with an apparent molecular weight of 77–78 kDa, is a membrane-bound flavoprotein containing one molecule of FMN and one molecule of FAD (Vermilion and Coon, 1974; Gum and Strobel, 1979). In CPR, the FAD receives two electrons from NADPH, while the FMN binding domain interacts with the P450 and reduces it (reviewed in Gutierrez *et al.*, 2003). CYP17A1 and CYP21A1 hydroxylation activities require two reducing equivalents. They are transferred upon direct interaction of P450 and CPR in the ER membrane (*Figure 11.4*). In order to avoid any possible transfer limitation of reducing equivalents to the microsomal steroid hydroxylases, it is possible either to improve expression of CPR or to elegantly fuse the CPR polypeptide to CYP17A1 or CYP21A1. Ohkawa and co-workers showed that it was possible to design functional fusion between CYP17A1 and CYP21A1 and *S. cerevisiae* P450 reductase (Ncp1p) (Sakaki *et al.*, 1990; Shibata *et al.*, 1990). These fusions were significantly more active in converting their respective substrate than the individual polypeptides *in vivo* and *in vitro*. On the contrary to the ADX, ADR couple, CPR from yeast is supple enough to allow either inter- or intra-molecular electron transfer in fusion polypeptides.

Another soluble electron transporter, namely cytochrome *b5* (cyt-*b5*), plays an apparently controversial role in steroidogenesis. Two types of cyt-*b5* (type 1 and type 2), sharing 45.8% of homology at the protein level, were discovered. Cyt-*b5*'s precise role is not yet clear. Studies suggest that cyt-*b5* can serve as an alternative electron donor, replacing CPR in some circumstances. Nevertheless, several studies demonstrated that cyt-*b5* type 1 and 2 selectively stimulate the CYP17A1 17,20-lyase activity by acting as allosteric effectors on the CYP17A1/CPR complex, facilitating electron transfer (Auchus *et al.*, 1998; Soucy and Luu-The, 2002). Interestingly, cyt-*b5* on its own cannot support catalysis (this has been reviewed in Miller, 2002). Unexpectedly, cyt-*b5* specifically stimulated cholesterol side-cleaving reconstituted activity, at least *in vitro*, by direct interaction with CYP11A1 in the presence of ADX and ADR (Chudaev *et al.*, 2001). The biological significance of this interaction is unknown, since cyt-*b5* and CYP11A1 are detected *in vivo* in cytosolic and mitochondrial compartments, respectively.

Why use yeast?

YEAST AS A MODEL ORGANISM

When one starts a project of reconstituting a pathway involving steroids into a recombinant host, there are general considerations related to the scale of genetic engineering that one must achieve on the one hand, and specific considerations

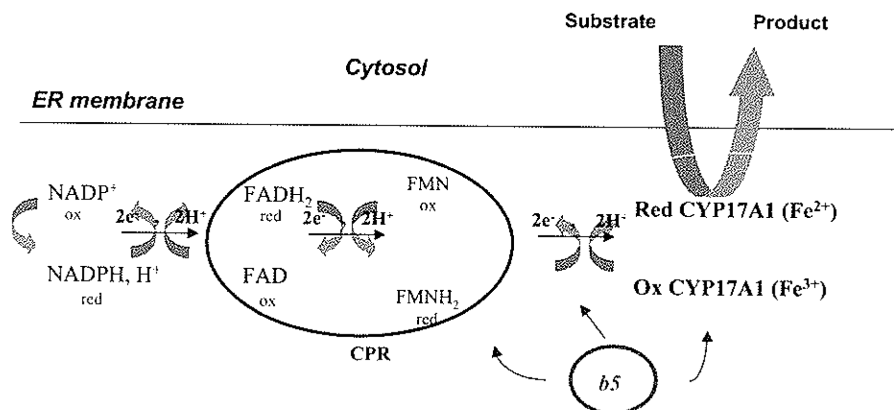


Figure 11.4. Scheme of electron transfer involved in microsomal P450 activities. CPR = NADPH cytochrome P450 oxidoreductase, *b5* = cytochrome *b5*, Ox = oxidized, Red = reduced.

related to steroids themselves with respect to their chemistry, as well as biological activity, on the other.

Genetic engineering is well developed in yeast, especially in *S. cerevisiae.* Decades of work renders budding yeast an organism of choice for the expression of multiple proteins in the same host. Furthermore, its genome was sequenced in 1996 (Goffeau *et al.*, 1996), and the annotation of the genome, relying on present and past scientific efforts to identify genes and their functions, is also now fairly advanced. At the present time, a large majority of the genes have been characterized and mutations in them can be linked to a phenotype (Scherens and Goffeau, 2004). This is, of course, an asset whenever one needs to modify or identify genes with an important or even detrimental activity. Moreover, a whole wealth of vectors for expression exists with different constitutive or inducible promoters (Bonneaud *et al.*, 1991; Schena *et al.*, 1991; Schneider and Guarente, 1991; Degryse *et al.*, 1995; Nacken *et al.*, 1996). Since *S. cerevisiae* possesses efficient homologous recombination machinery, it is possible to introduce expression cassettes by homologous recombination into a specific chosen region. Another possibility is to use multicopy vectors based on the yeast endogenous 2-micron replication origin or low copy (one or two copies per cell) based on autonomously replicating and centromeric sequences (ARS/CEN). Used in conjunction with these episomal vectors, any genome modification is possible, provided it does not interfere with essential gene function. The expression cassette can be introduced either inter-genetically or intragenetically (i.e. inside a gene). In the first instance, the expression cassette is introduced in between two genes, one of them being the selection marker, so that the functionality of the respective promoters is maintained. In the second instance, the non-essential gene is disrupted sequentially in order to replace its coding sequence by a functional expression cassette.

Associated with different promoter strength, these complementary ways of introducing genes into *S. cerevisiae* allow expression at different levels and favourable conditions, which can be the key to obtaining a correct expression and coordination

of multiple activities. So, yeast, in particular *S. cerevisiae*, offers the possibility to express multiple proteins in the same organism.

INTERESTING FEATURES OF *S. CEREVISIAE* FOR THE EXPRESSION OF CYTOCHROME P450S

Microsomal P450 expression and function rely exclusively on ubiquitous electron carriers, which are transferring electrons to the enzyme in order to split molecular oxygen. In mammals, a specific protein NADPH P450 reductase from the endoplasmic reticulum is capable of doing so (see above). Interestingly, in *S. cerevisiae*, a protein Ncp1p is capable of efficiently replacing the *bona fide* CPR. It is capable of providing electrons to exogenous P450 expressed without impairing the *S. cerevisiae* life cycle (Sakaki *et al.*, 1989, 1991; Gautier *et al.*, 1993). Its physiological role is to provide electrons to P450 involved in the ergosterol biosynthesis, namely Erg11p (CYP51, lanosterol 14alpha-demethylase) and Erg5p (delta 22 sterol desaturase) genes and to Dit2p (second enzyme in the pathway for biosynthesis of dityrosine in the outer layer of the spore wall). *ERG11* and *ERG5* gene products are essential for the production of ergosterol, but the electron flux provided by Ncp1p appears to be in excess and sufficient to provide electrons to exogenous P450s. In other words, over-expression of exogenous P450 at reasonable levels should not impair the *S. cerevisiae* life cycle. Regardless, it is always possible using the appropriate expression vectors to over-express NCP1 in order to avoid any interference with the *S. cerevisiae* life cycle (Gautier *et al.*, 1993).

A few mitochondrial P450, especially those implicated in the steroid hormone synthesis of mammals, use different electron carriers, ADX and ADR, which were described, above. Unexpectedly, ADR can be efficiently replaced by an ADR related homologue or Arh1p from *S. cerevisiae* (Dumas *et al.*, 1996). Arh1p is essential for *S. cerevisiae* and involved in iron homeostasis (see above).

In summary, *S. cerevisiae* expresses two electron carriers, Ncp1 and Arh1p, that are an asset for expression of mammalian cytochrome P450s.

ROUTING THE ERGOSTEROL PATHWAY IN *S. CEREVISIAE*

Fungi present ergosterol as a main sterol in their membrane, as ergosterol is the end product of the sterol pathway. Interestingly, enzymes implicated in the latter part of the pathway are not essential for *S. cerevisiae*. For example, genes corresponding to the enzymes Erg6p (S-adenosylmethionine delta-24-sterol-C-methyltransferase) (Gaber *et al.*, 1989), Erg5p (Cytochrome P450, delta 22(23) sterol desaturase) (Hata *et al.*, 1983), Erg2p (Sterol C8–C7 isomerase (C-8 sterol isomerase)) (Palermo *et al.*, 1997), Erg3p (encoding the C-5 sterol desaturase enzyme) (Palermo *et al.*, 1997), and Erg4p (Sterol C-24 (28) reductase) (Zweytick *et al.*, 2000), respectively, are not essential in all the *S. cerevisiae* strains that have been tested so far. The membrane composition of *S. cerevisiae* is flexible enough to afford modifications of the insaturations of the B-ring (Erg2p and Erg3p) or of the side-chain (Erg4p, Erg5p, and Erg6p).

In mammals and yeast, cholesterol and ergosterol are major components of their membranes, respectively. Ergosterol differs from cholesterol because of the presence

of two double bonds; one 7(8) on the B-ring of the backbone and the second 22(23), together with a methyl at position 24. A few reactions could modify ergosterol into molecules resembling cholesterol. So, there are two possibilities to transfer a functional cholesterol metabolizing pathway, either construct strains producing molecules mimicking cholesterol or design mutants that are capable of taking up cholesterol. *S. cerevisiae* is inefficient for the uptake of sterols in aerobiosis conditions where P450s can be functional. Most of the mutants efficiently taking up cholesterol concern the heme biosynthesis, which is *a priori* unfavourable for the expression of heme bearing proteins, such as P450. Some mutants, such as upc2, showing a normal heme synthesis and having an improved capacity to take up sterol, were described (Crowley *et al.*, 1998). This mutant had a small sterol uptake compared to mutants in the heme biosynthesis pathway. Attempts to express P450 proteins, such as CYP17A1, in heme deficient strains failed, even in conditions complementing the heme biosynthesis pathway (Ness *et al.*, 1998). In conclusion, *S. cerevisiae* offers many advantages for the expression of a complex set of membrane proteins forming a pathway. The possibility of generating strains making proper sterols for CYP11A1 has to be explored.

Transferring the hydrocortisone pathway in a single recombinant organism

S. cerevisiae was chosen as a host for transferring this complex pathway because, on the one hand, its fermentation is easily industrialized and, on the other hand, its physiology is well characterized and a majority of its genes have a known function. Finally, numerous tools and mutants are available, together with the possibility of using molecular genetics for combining an appropriate set of genes. We will present here the transfer in the order of the enzymatic steps along the pathway.

The first reaction to be transferred in yeast is the side-chain cleaving of cholesterol into pregnenolone. As explained above, this reaction needs a good balance between substrate concentration, CYP11A1, ADX, and ADR expression level. Substrate cholesterol, mostly from bovine origin or phytosterol from plant, is a hurdle because it is poorly soluble and it is difficult to make it available at the site of CYP11A1 expression. The second hurdle is that, even if mutants are designed for taking up sterol, they will probably be incompatible for side-chain cleaving activity of the CYP11A1, ADX, and ADR complex since this requires molecular oxygen, and cholesterol uptake is the best in anaerobiosis (Ness *et al.*, 1998). The third hurdle is that, after being taken up by yeast, cholesterol will be esterified by Are1p and Are2p, the two proteins that are responsible for esterification of ergosterol and its precursors (Parks and Casey, 1995; Yang *et al.*, 1996). It is difficult to infer if the cholesterol that has been taken up in this way will be available for cleavage by CYP11A1. These observations lead to the conclusion that it might be easier to use endogenous sterols, even if they are rather poor substrates for CYP11A1. Moreover, CYP11A1 appears to show some flexibility with regards to the side-chain specificity of its substrate (Morisaki *et al.*, 1980; Tuckey and Cameron, 1993b).

Little is known about the specificity of CYP11A1 towards the cholesterol backbone, even though it has been shown recently that CYP11A1 could metabolize 7-dehydro cholesterol with the same efficiency as cholesterol (Guryev *et al.*, 2003). Expressing in a suitable host a plant Δ7-reductase, elegantly cloned by metabolic

interference using nystatin, can reduce the 7–8 double bond – the main difference between ergosterol and cholesterol. This latter molecule is a classical anti-fungal compound known to interact with the 7–8 double bond of ergosterol (Lecain *et al.*, 1996). It would then be possible to generate strains able to produce as major sterols either ergosta-5-enol and ergosta-5-22-dienol, or ergosta-5-enol alone, whenever the sterol Δ22 desaturase gene (*ERG5*) was disrupted (*Figure 11.2*) (Duport *et al.*, 1998).

To obtain a functional side-chain cleavage activity, it is necessary to express two polypeptides if targeted to the mitochondria, or three polypeptides if targeted outside the mitochondria. In the former situation, the chain will be NADPH, ADR, ADX, and CYP11A1, probably in the ER; in the latter, the electrons will be transferred from NADPH to Arh1p (the *S. cerevisiae* ADR homologue), ADX, and CYP11A1 in the mitochondria. In order to diminish the competition for reducing equivalents in the mitochondria in the case of coexpression of CYP11A1 and CYP11B1, and to avoid the problem of bringing the substrate to the inner mitochondria that is poor with respect to its sterol content, we have chosen to express the mature form of the above polypeptides in a host producing ergosta-5-enol and ergosta-5-22 dienol, or ergosta-5-enol alone (*Figure 11.2*, white panel) (Duport *et al.*, 1998). To our surprise, a strain expressing the three peptides and phytosterols was seen to produce *in vivo* pregnenolone, together with an esterified form of pregnenolone, later identified by Cauet and co-workers as acetylpregnenolone (Duport *et al.*, 1998; Cauet *et al.*, 1999) (*Figure 11.2*, white dotted panel).

Interestingly, *S. cerevisiae* produces a protein that is capable of efficiently metabolizing pregnenolone into acetylpregnenolone, thus preventing any further metabolism by 3β-HSD as the position 3 is blocked (Cauet *et al.*, 1999). Using classical biochemical techniques, it has been possible to identify the protein involved in this efficient esterification as an alcohol O-acetyl transferase named Atf2p (Cauet *et al.*, 1999). In a more recent study, Atf2p was found responsible for the formation of volatile esters in yeast (Verstrepen *et al.*, 2003). Disruption of the corresponding gene totally abolished the yeast capacity to esterify pregnenolone (Cauet *et al.*, 1999). It was possible to introduce in strains wild type for *ATF2* expressing an active side-chain cleaving system, an expression cassette for 3β-HSD, as it was shown to be active in yeast (Degryse *et al.*, 1999). Part of the free pregnenolone was then transformed into progesterone (Duport *et al.*, 1998).

Lecain and co-workers have demonstrated that it is possible to re-route the yeast sterol into making phytosterol using a plant sterol Δ7-reductase (Lecain *et al.*, 1996). These sterols are further metabolized into pregnenolone using a non-mitochondrial side-chain cleaving enzyme (Duport *et al.*, 1998). This pregnenolone is further transformed into acetylpregnenolone by a yeast enzyme that has been characterized (Cauet *et al.*, 1999). Thus, the sterol Δ7-reductase activity is linking the yeast pathway with the mammalian hydrocortisone pathway by transforming ergosterol and its derivatives into sterols compatible with CYP11A1 (connected dark and light grey panel on *Figure 11.2*). By adding a copy of 3β-HSD, it is possible to metabolize free pregnenolone into progesterone (Duport *et al.*, 1998) (*Figure 11.2*, grey panel).

It has also been shown that it is possible to link the 3β-HSD reaction and the hydroxylation reaction at position 17 (Degryse *et al.*, 1999) on the one hand, and

the hydroxylation reactions at positions 17 and 21 (Sakaki *et al.*, 1991) on the other hand. The balance between the corticosterone pathway and the 11-deoxycortisol pathway should be achieved by the correct balancing of 3β-HSD, CYP17A1, and CYP21A1 (Sakaki *et al.*, 1991; Degryse *et al.*, 1999; Kominami *et al.*, 2001). So, it appears that it is possible to introduce in the genome of yeast strains an expression cassette containing the CYP17A1 cDNA under the control of a strong yeast promoter (Szczebara *et al.*, 2003). To our surprise, in experiments using galactose as a carbon source, the transformed strain was seen to produce very little 17α-hydroxyprogesterone but a more polar product that was identified as 17α,20α-dihydroxypregn-4-ene-3-one (Szczebara *et al.*, 2003). Moreover, we were able to show that wild type yeast, when incubated with 17α-hydroxyprogesterone, could transform this steroid into 17α,20α-dihydroxypregn-4-ene-3-one (Dumas *et al.*, 1994; Shkumatov *et al.*, 2002). It is interesting to note that this activity was seen to seriously hinder the capacity of CYP21A1 to transform 17α-hydroxyprogesterone in yeast *in vivo* (Szczebara *et al.*, 2003) (*Figure 11.2*, bottom right panels).

A 20α-hydroxysteroid dehydrogenase (20α-HSD) activity has been reported in mammalian endocrine tissue (Zhang *et al.*, 2000) and has led us to identify appropriate homologues in yeast. In the yeast genome, a family of six genes was found to have significant identities with the cloned mammalian cDNA. Among these six corresponding proteins, Gcy1p and Ypr1p have been described as the best candidates because of their highest identity, respectively 44 and 43%, with the described bovine protein (Szczebara *et al.*, 2003). *GCY1* is a galactose-regulated gene, so was most of the *S. cerevisiae* 20α-HSD activity (Szczebara *et al.*, 2003). Disruption of *GCY1* and *YPR1* totally abolished the 20α-HSD activity of WT yeast (Szczebara *et al.*, 2003). Upon disruption of these two genes and expression of recombinant CYP21A1, it was possible to recover up to 70% of 11-deoxycortisol in a bioconversion experiment from 17α-hydroxyprogesterone (Szczebara *et al.*, 2003). So, it is apparent that a strain linking the reaction from phytosterols to hydrocortisone should be devoid of Gcy1p and Ypr1p activities.

To synthesize hydrocortisone, one must be able to transform 11-deoxycortisol into hydrocortisone. It is necessary to express in the same organelle three polypeptides, namely ADX, ADR, and CYP11B1. The choice is limited either to target the polypeptides into yeast mitochondria or to use mature forms of the three polypeptides. There are no examples in the literature of functional expression of CYP11B1 or CYP11B2 as mature polypeptides, while ADX and ADR are functionally expressed as mature polypeptide in yeast (Akiyoshi-Shibata *et al.*, 1991; Duport *et al.*, 1998). CYP11B1 and CYP11B2 are difficult to manipulate and express. So far, the only way to detect their functions either *in vitro* or *in vivo* is to target them to yeast mitochondria, either in *S. cerevisiae* or in *S. pombe* (Dumas *et al.*, 1996; Bureik *et al.*, 2002b; Szczebara *et al.*, 2003). Interestingly in both cases, the *in vivo* activity relies on endogenous electron carriers, which were identified (Lacour *et al.*, 1998; Schiffler *et al.*, 2004a). In *S. pombe*, the natural CYP11B2 polypeptide (with its mammalian targeting pre-sequence) is targeted to mitochondria and receives electrons from an endogenous adrenodoxin-like ferredoxin, which has been characterized. The endogenous ADR-like homologue has not been characterized. In other words, a single polypeptide is enough to reconstitute the CYP11B2 activity in *S. pombe* (Bureik *et al.*, 2002b). The situation is different in *S. cerevisiae*, where the

expression of two polypeptides is necessary to observe conversion of 11-deoxycortisol into hydrocortisone (Dumas *et al.*, 1996). The adrenodoxin reductase homologue Arh1p is endogenous to the strain; it was isolated and characterized, while the exogenous polypeptides are ADX and CYP11B1 targeted to mitochondria.

Finally, with the goal of constituting a recombinant hydrocortisone pathway, it has been demonstrated that it is possible to link the yeast sterol pathway with the first steps of the hydrocortisone pathway to produce progesterone, provided the gene encoding endogenous acetyl ester synthase, *ATF2* gene, is disrupted. Linking of 3β-HSD with 17-hydroxylase activity (Degryse *et al.*, 1999) and linking 17-hydroxylase activity with 21-hydroxylase activity (Sakaki *et al.*, 1991) are both possible.

Independently, 11-deoxycortisol can be transformed into hydrocortisone by recombinant yeast expressing CYP11A1 and its partner ADX (Dumas *et al.*, 1996).

All the reactions of the pathway have thus been shown to work individually: the next stage is to reconstitute them in a single host. A schematic overview of what we propose should be done is provided in *Figure 11.5*. On panel A, the genes and cDNAs that should be expressed, tuned or disrupted are listed, while on panel B, the yeast tools to perform these tasks are cartooned. In order to obtain a single cell making hydrocortisone from endogenous sterols, one needs to express 9 proteins, namely: sterol Δ7-reductase; mature forms of ADX, ADR, and CYP11A1; 3β-HSD, CYP17A1, CYP21A1, mitochondrially targeted ADX, and CYP11B1. That is to say that for each protein, an individual expression cassette should be constructed containing a suitable promoter, either 'constitutive' or regulatable, the cDNA of interest, and a transcriptional terminator.

The two main ways of regulating the expression level of a recombinant protein are by using promoters of variable strength and temporal expression and by varying the copy number. For example: *TDH3*prom, *CYC1*prom, and *GAL10*prom are three different yeast promoters. *TDH3*prom is a strong constitutive promoter, while *CYC1*prom is a weak promoter (Nacken *et al.*, 1996). *GAL10*prom is a strong galactose inducible promoter (Guarente *et al.*, 1982). Expression is also tuned with the expression cassette copy number. One can use a single expression cassette integrated in the genome either inside an existing gene (*Figure 11.5b*: 1) or in between two genes (*Figure 11.5b*: 2). An almost equivalent expression system exists with an episomal vector based on an ARS/CEN sequence; in this case, no integration in the genome is necessary (*Figure 11.5*, LC: low copy number plasmid). In general, higher expression levels can be achieved using multi-copy plasmid based on a 2-micron replication origin (between 20 to 100 copies/cell; *Figure 11.5*, HC: high copy number plasmid). Provided that the recombinant protein in question is stable enough, its expression can be modulated at will. Two other operations should be performed before completing the transfer: tuning the expression level of endogenous protein, and disrupting unwanted reactions. In the present case of interest, namely Ncp1 or Arh1p, the electron providers for microsomal and mitochondrial P450s, respectively, should be tuned as it is essential to have an optimized flux of electrons towards the hydroxylating enzymes (Gautier *et al.*, 1993; Kominami *et al.*, 2001; Schiffler *et al.*, 2001). This can be accomplished either by changing the natural promoter or adding a second copy of these genes

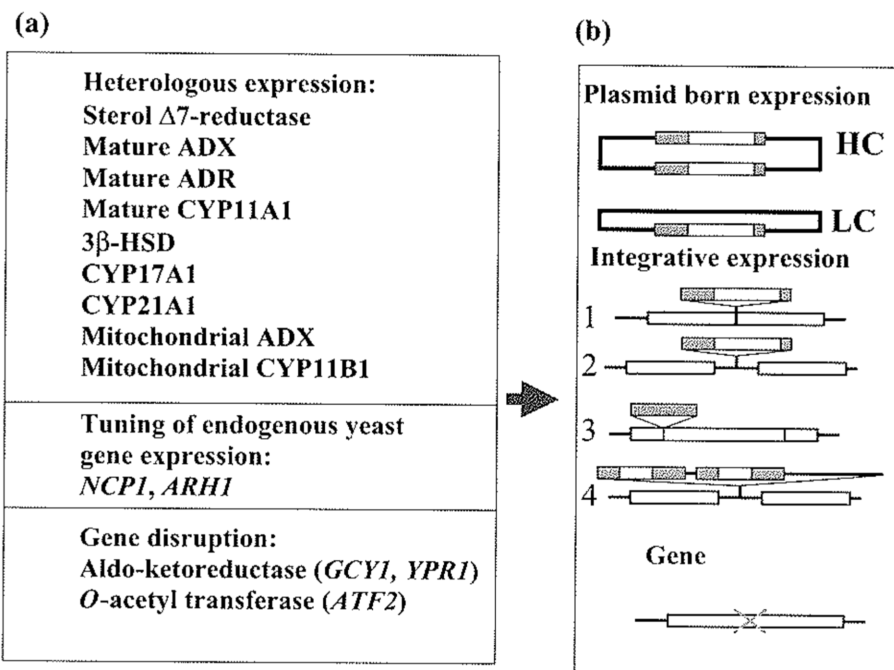


Figure 11.5. *S. cerevisiae* 'juggling' for tuning of the recombinant pathway. a) Expression, tuning of endogenous genes, gene disruption. b) *S. cerevisiae* tools: HC, high copy; LC, low copy. 1) Intragenic expression; 2) intergenic expression; 3) genomic modification; 4) duplication of an existing gene.

(*Figure 11.5b*: 3 and 4) (Gautier *et al.*, 1993). In order to get an optimized flux of the pathway towards hydrocortisone, it is imperative to get rid of the genes responsible for pregnenolone acetylation (*ATF2*) and 20 keto-reduction (*GCY1, YPR1*). This can be achieved by deletion–insertion through homologous recombination of selectable markers into the corresponding genes, i.e. *ATF2, GCY1*, and *YPR1*. Szczebara and co-workers proposed a solution to this impressive puzzle (Szczebara *et al.*, 2003). In order to drive the metabolism towards hydrocortisone production, mature CYP11A1, mitochondrial CYP11B1, and mature ADX (corresponding to the two ends of the pathway) were expressed at high level on a multi-copy plasmid, whilst mature forms of ADR and 3β-HSD were produced from a low copy number plasmid. CYP17A1, CYP21A1, mitochondrial ADX, and sterol Δ7-reductase cDNAs were integrated at various spots in the genome. The balance between CYP17A1 and CYP21A1 was tuned in favour of CYP17A1 in order to avoid the accumulation of corticosterone through formation of 17-hydroxypregnenolone. Arh1p was overproduced in order to avoid a potential limitation of electron flux towards Yah1p (the partner of Arh1p) (Barros *et al.*, 2002) and ADX. A strain expressing the nine proteins of the pathway (as described above) where the unwanted reactions are disrupted was able to produce steroids, of which 70% were hydrocortisone (Szczebara *et al.*, 2003). The other steroids produced in significant amounts were corticosterone and 11-deoxycortisol (Szczebara *et al.*, 2003). Getting rid of these intermediates is a matter of balancing CYP17A1 and CYP21A1 (in order to avoid accumulation of deoxycorticosterone)

and improving the 11β-hydroxylation reaction (in order to avoid accumulation of 11-deoxycortisol).

In this report, we have reviewed the elements that are necessary to produce hydrocortisone from a simple carbon source by recombinant *S. cerevisiae* strains. The major hurdles approached in the course of such a transformation are:

(1) production of suitable sterols for CYP11A1;
(2) expression of the mitochondrial P450 and their electron carriers in a suitable environment;
(3) identification and elimination of host-related unwanted reactions;
(4) channelling the metabolic flux;
(5) toxicity of certain biosynthetic intermediates; and
(6) appropriate juggling with the genetic engineering of yeast.

Because aerobic uptake of exogenous sterols by yeast is hindered, the natural endogenous ergosterol pathway (endogenous pathway) was re-routed in order to produce molecules resembling cholesterol. A functional plant enzyme was used to reduce the 7–8 double bond of the ergosterol backbone, rendering the sterol obtained suitable for the side-cleaving reaction (white panel of *Figure 11.2*). The two-mitochondrial steps caused major problems (CYP11A1 and CYP11B1). The former was reconstituted outside of the mitochondria using mature polypeptides, while the latter, relying in part on the host electron transfer capacity, was targeted to the mitochondria. Interestingly, CYP11A1 has been detected mostly at the plasma membrane (Duport *et al.*, 2003), whilst its partners, ADX and ADR, have been detected as a cytosolic and microsomal protein, respectively (Akiyoshi-Shibata *et al.*, 1991). So, electron transfer probably can take place between a plasma membrane protein and a protein from the endoplasmatic reticulum. It is not known whether this unexpected finding has a profound influence on the side-chain cleaving reaction. On the contrary, CYP11B1 reaction is taking place in the inner mitochondrial membrane of *S. cerevisiae* (Dumas *et al.*, 1996; Lacour *et al.*, 1998; Cauet *et al.*, 2001).

Alongside these developments, two parasitic reactions have also been identified: acetylation of pregnenolone performed by the *ATF2* gene product (Cauet *et al.*, 1999), and a 20-ketoreduction encoded by the *GCY1* and *YPR1* gene products (Szczebara *et al.*, 2003). Whilst the *GCY1* and *YPR1* gene family was very quickly recognized because of the existence of a mammalian equivalent, *ATF2* was much more difficult to identify because its function was unexpected and uncharacterized. Moreover, this protein appears to be expressed at a relatively low level and is sensitive to protease degradation (Cauet *et al.*, 1999). The three genes coding for these parasitic reactions could be disrupted using a classical dominant marker, or in a more sophisticated way using expression cassettes coding for CYP21A1.

Finally, the eight proteins (namely, mature forms of ADX, ADR, and CYP11A1, mitochondrial forms of ADX and CYP11B1, 3β-HSD, CYP17A1, and CYP21A1) of the mammalian pathway can be simultaneously and functionally expressed in the modified host containing phytosterols compatible with CYP11A1 (Szczebara *et al.*, 2003). This has allowed the detection of hydrocortisone and other steroids in the culture broth of the strains (Szczebara *et al.*, 2003). The most advanced strain with deletion of the *GCY1*, *YPR1*, and *ATF2* genes have been found to produce

hydrocortisone as their main steroid. The other steroids are proximal intermediates of hydrocortisone, i.e. corticosterone and 11-deoxycortisol. As in the mammalian cells, the balance of corticosterone over hydrocortisone is in favour of hydrocortisone. Corticosterone accumulation indicates a balance between CYP17A1 and CYP21A1 in favour of CYP21A1. To avoid accumulation of corticosterone, the product of 21-hydroxylation of 17-hydroxypregnenolone, one must increase the CYP17A1 expression level to favour the production of 17-hydroxyprogesterone. The accumulation of 11-deoxycortisol can be avoided by improving the capacity of the 11β-hydroxylation reaction. The regulation of the natural hydrocortisone pathway is not known, but it is generally accepted that the side-chain cleavage reaction is its limiting step (Miller, 1988). It is not known whether this is the case for the recombinant pathway of *S. cerevisiae*. Interestingly, the effect of pregnenolone on *S. cerevisiae*, i.e. accumulation of sterol biosynthesis intermediates (Duport *et al.*, 2003), is not observed corroborating the absence of pregnenolone accumulation in the UCY strains (Szczebara *et al.*, 2003).

Five yeast genes (*ATF2*, *GCY1*, *YPR1*, *ARH1*, and *NCP1*) have a decisive influence on the steroid pathway. Genes such as *ATF2*, *GCY1*, and *YPR1* modify or impair the proper flux of steroids towards hydrocortisone, while some other genes and their gene products, such as *NCP1* and *ARH1*, are fundamental for the life cycle of *S. cerevisiae* and steroid production. Among these five genes, *ATF2* has the most profound influence by sequestering pregnenolone from being further metabolized. This was an unexpected finding since free pregnenolone is a potent inhibitor of sterol biosynthesis (Duport *et al.*, 2003).

In conclusion, it is possible to engineer strains to produce hydrocortisone from glucose (or ethanol) with a good yield. *ARH1*, *ATF2*, *GCY1*, and *YPR1* endogenous genes appear to play a fundamental role for the correct functioning of the heterologous pathway. The hydrocortisone biosynthesis pathway, although not perfectly balanced, appears to lead significantly to the accumulation of, besides hydrocortisone, two other key intermediates, namely corticosterone and cortexolone. The strains described in this report would, in our opinion, provide a magnificent tool for deciphering the hydrocortisone pathway and, in particular, to study this balance. However, whilst these recombinant strains mimick the mammalian pathway, contrary to the mammalian situation, CYP11A1 is probably targeted to the plasma membrane. Somehow, the recombinant yeast strains are keeping intrinsic regulation, as shown by the profound effect of *ATF2* deletion.

It can be reasonably inferred that this technology will permit not only the production of cheaper steroids but also provides a way of deciphering the mammalian steroid biosynthesis. It should allow us to study the balance between the different steroids in a systematic fashion. This balance, which is an essential component of mammalian life, can be assessed by introducing different perturbations through modifications of key protein expression levels using genetics (promoter strength, RNA interference, gene disruption, or removal) or drugs (inhibitors of key enzymes).

Acknowledgements

We are indebted to the current UCI and Process Development Biotechnology teams

for continuous support. This work would not exist without the encouragement of K. Assemat-Lebrun and P. Baduel. Our thanks go to I. Maury, C. Marcireau, M.F. Paul, O. Dos-Santos, G. Simoes, and A. Fournier for their attention during the completion of this work. Prof T. Achstetter has been of great help by reading the manuscript.

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