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Cytochromes P450 as versatile biocatalysts

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Abstract

Cytochromes P450 are ubiquitously distributed enzymes, which were discovered about 50 years ago and which possess high complexity and display a broad field of activity. They are hemoproteins encoded by a superfamily of genes converting a broad variety of substrates and catalysing a variety of interesting chemical reactions. This enzyme family is involved in the biotransformation of drugs, the bioconversion of xenobiotics, the metabolism of chemical carcinogens, the biosynthesis of physiologically important compounds such as steroids, fatty acids, eicosanoids, fat-soluble vitamins, bile acids, the conversion of alkanes, terpenes, and aromatic compounds as well as the degradation of herbicides and insecticides. There is also a broad versatility of reactions catalysed by cytochromes P450 such as carbon hydroxylation, heteroatom oxygenation, dealkylation, epoxidation, aromatic hydroxylation, reduction, dehalogenation (Sono, M., Roach, M.P., Coulter, E.D., Dawson, J.H., 1996. Heme-containing oxygenases. Chem. Rev. 96, 2841–2888), (Werck-Reichhart, D., Feyereisen, R., 2000. Cytochromes P450: a success story. Genome Biol. 1 (REVIEWS3003)), (Bernhardt, R., 2004. Cytochrome P-450. Encyclopedia Biol. Chem. 1, 544–549), (Bernhardt, R., 2004. Optimized chimeragenesis; creating diverse P450 functions. Chem. Biol. 11, 287–288), (Guengerich, F.P., 2004. Cytochrome P450: what have we learned and what are the future issues? Drug Metab. Rev. 36, 159–197). More than 5000 different P450 genes have been cloned up to date (for details see: http://drnelson.utmem.edu/CytochromeP450.html). Members of the same gene family are defined as usually having ≥40% sequence identity to a P450 protein from any other family. Mammalian sequences within the same subfamily are always >55% identical. The numbers of individual P450 enzymes in different species differ significantly, showing the highest numbers observed so far in plants.

The structure–function relationships of cytochromes P450 are far from being well understood and their catalytic power has so far hardly been used for biotechnological processes.

Nevertheless, the set of interesting reactions being catalysed by these systems and the availability of new genetic engineering techniques allowing to heterologously express them and to improve and change their activity, stability and selectivity as well as the increasing interest of the industry in life sciences makes them promising candidates for biotechnological application in the future.

Keywords: Cytochrome P450; Biotechnology; Biocatalysis; Regio- and stereoselective hydroxylation; Steroid hydroxylation; CYP101; CYP102; CYP106A2

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1. General aspects

Cytochromes P450 are external monooxygenases. Monooxygenases (mixed function oxidases) catalyse the incorporation of a single atom of molecular oxygen into a substrate with the concomitant reduction of the other atom to water. The monooxygenases are divided into two classes, the internal and the external monooxygenases. Internal monooxygenases extract two reducing equivalents from the substrate to reduce one atom of dioxygen to water, whereas external monooxygenases utilize an external reductant (see Ruckpaul et al., 1989). While initially the microsomal drug and xenobiotic-metabolising enzymes were referred to as mixed function oxidases, in more recent years the term monooxygenase became the more accepted one.

Cytochromes P450 got their name from their character as hemoproteins and from their unusual spectral properties displaying a typical absorption maximum of the reduced CO-bound complex at 450 nm: cytochrome stands for a hemoprotein, P for pigment and 450 reflects the absorption peak of the CO complex at 450 nm. The ability of reduced P450 to produce an absorption peak at 450 nm upon CO binding is still used for the estimation of the P450 content (Omura and Sato, 1964). The red shift of about 30 nm as observed in cytochromes P450 means that the distribution of electron density at the heme is significantly perturbed as compared to other cytochromes. It has been documented that the cause of this is the thiolate sulphur, which by means of a direct bond to the iron causes this effect. The Soret band (named after its discoverer) describes the absorption band of hemoproteins at about 380–420 nm.

Cytochrome P450 systems catalyse the following reaction:

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

They are involved in reactions as diverse as e.g. hydroxylation, N-, O- and S-dealkylation, sulphoxidation, epoxidation, deamination, desulphuration, dehalogenation, peroxidation, and N-oxide reduction (Fig. 1). Dawson and coworkers (Sono et al., 1996) list more than 20 different reactions, which can be catalysed by cytochromes P450. Some more unusual reactions were recently summarised by (Guengerich, 2001a, b). Their substrates include fatty acids, steroids, prostaglandins, as well as a multitude of foreign compounds such as drugs, anaesthetics, organic solvents, ethanol, alkylaryl hydrocarbon products, pesticides, and carcinogens. This diversity of catalysed reactions and acceptable substrates attracted researchers from different fields to study cytochrome P450 systems. Besides pharmacologists and toxicologists also endocrinologists, physiologists, microbiologists, organic chemists, plant biologists and environmental scientists are working on diverse aspects of P450 function and regulation.

Hydrocarbon hydroxylation
Alkene epoxidation
Alkyne oxygenation
Arene epoxidation
Aromatic hydroxylation
N-Dealkylation
S- Dealkylation
O- Dealkylation
N-Hydroxylation
N-Oxidation
S-Oxidation
Oxidative deamination
Oxidative dehalogenation
Alcohol and aldehyde oxidations
Dehydrogenation
Dehydratations
Reductive dehalogenation
N-Oxide reduction
Epoxide reduction
Reductive β-scission of alkyl peroxides
NO reduction
Isomerizations
Oxidative C-C bond cleavage

Fig. 1. Reactions catalysed by cytochromes P450, according to (Sono et al., 1996).

2. Chemistry of oxygen activation: the catalytic cycle

The structure and chemistry of cytochromes P450 have very recently been summarised in an excellent review (Denisov et al., 2005) and therefore will not be discussed here in detail. In general, the catalytic turnover of cytochromes P450 starts with the binding of substrate followed by the introduction of the first electron from NAD(P)H via an electron transfer chain. In the next step, oxygen binds, which is capable of

accepting the second electron to produce a ferric peroxy anion. In the next steps the ferric peroxy anion is protonated to form the ferric hydroperoxy complex, which undergoes subsequent heterolytic cleavage with the formation of a putative ferryl species, formally equivalent to a Fe(V)=O species named Compound I. This species (or a similar reactive, electrophilic iron-oxo intermediate) then attacks the substrate, yielding the hydroxylated product, which dissociates to let the cycle start again. There are two major abortive reactions: (i) autoxidation of the oxy-ferrous enzyme with concomitant production of a superoxide anion and (ii) oxidase uncoupling with oxidation of the ferryl-oxo intermediate to water by a four-electron reduction of the dioxygen molecule. In the absence of a suitable substrate, some reduced P450 enzymes very efficiently produce reactive oxygen species (ROS) by autoxidation in vitro and in vivo, which in mammals can lead to the induction of apoptosis (Nordblom et al., 1976; Nordblom and Coon, 1977; Hanukoglu et al., 1993; Liu et al., 2003; Derouet-Humbert et al., 2005).

Interestingly, in many, but not all, P450s also a so-called "shunt" reaction can proceed, where the substrate can be hydroxylated directly by peroxides such as hydrogen peroxide, cumene hydroperoxide and *tert*-butyl hydroperoxide without the necessity of stepwise activation of molecular oxygen and an interaction with electron donating systems. This "peroxygenase" activity offers an opportunity to utilize cell-free P450-dependent catalysis without the necessity of NAD(P)H regeneration, additional proteins or dioxygen.

Most reactions catalysed by P450s appear to be consistent with this catalytic cycle.

Although the general reaction cycle of cytochromes P450 became clear in the early 1980s (White et al., 1980), for reviews see also (Porter and Coon, 1991; Guengerich, 2001a,b, 2002, 2004; Denisov et al., 2005), it was not until about 1990 that the mechanism of oxygen activation got deciphered, at least for CYP101 (P450cam), where most of the mechanistic studies have been performed (Poulos et al., 1987; Imai et al., 1989; Imai and Nakamura, 1989; Raag and Poulos, 1991; Raag et al., 1991; Gerber and Sligar, 1992; Kimata et al., 1995; Schlichting et al., 2000).

Taken together, cytochromes P450 do not only catalyse monoxygenase, but also oxidase and peroxidase reactions. Variations of this scheme for the reaction mechanism of P450, however, occur with different P450 systems like thromboxane and prostacyclin synthase, nitric oxide reductase (CYP55A1) and others. These different reaction types are, in addition to the main reaction cycle, the basis for the versatility of cytochromes P450.

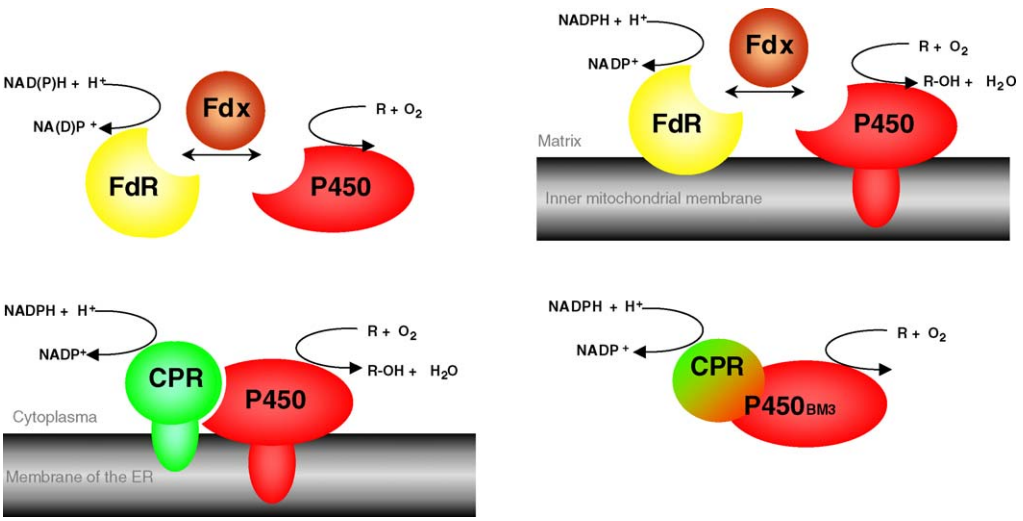

Fig. 2. Schematic organisation of different cytochrome P450 systems. Upper row, left: bacterial system, right: mitochondrial system. Lower row, left: microsomal system, right: self-sufficient CYP102 (P450-BM3).

3. Structural organization of cytochrome P450 systems

As mentioned above, cytochromes P450 belong to external monooxygenases (for review cf. Bernhardt, 2004a, b). This implicates that they need an external electron donor, which transfers the electrons necessary for oxygen activation and the following substrate hydroxylation. Two main classes of cytochromes P450 with respect to their electron supporting system can be defined, although other subclasses are occurring, too: the mitochondrial/bacterial type and the microsomal type (Fig. 2). Microsomal cytochromes P450 are membrane bound and accept electrons from a microsomal NADPH-cytochrome P450 reductase, containing FAD and FMN. All drug and xenobiotica metabolising cytochromes P450 isolated so far and, in addition, CYP102 (P450BM-3) isolated from *Bacillus megaterium* were shown to belong to this class. This latter P450 system consists of a polypeptide chain with two different domains, one containing the hemoprotein and the other one containing a FAD-reductase. Most of the other bacterial cytochromes P450 belong to the second class. They are soluble and obtain the electrons necessary for the reaction mechanism from an NADH-dependent FAD-containing reductase via an iron–sulphur protein of the [2Fe–2S] type. Mitochondrial cytochromes P450 being involved in the side-chain cleavage of cholesterol, the 11β-hydroxylation of 11-deoxycortisol, the production of aldosterone and in vitamin D biosynthesis also belong to the latter class. These cytochromes P450 are localized in the inner mitochondrial membrane, whereas the [2Fe–2S] protein, called adrenodoxin in the case of adrenal steroid hydroxylase systems, is a soluble protein of the matrix. The FAD-containing reductase, adrenodoxin reductase (AdR), is associated with the inner mitochondrial membrane.

Interaction of the cytochromes P450 with their corresponding electron donors is a necessary prerequisite of the catalytic cycle. Its specificity guarantees a sufficient reaction rate of catalysis and likewise a discrimination between different potential donors and acceptors of electrons to protect the system from shunt reactions.

Since in liver microsomes many different isoenzymes have to interact with only one type of reductase it has to be expected that the binding site for reductase is very similar or identical on various cytochromes P450. Salt bridges are responsible for the recognition of the reductase by the P450 and the correct orientation of both proteins to each other (for review cf. Bernhardt, 1993; Bernhardt, 1996). In addition to microsomal reductase, some microsomal cytochromes P450 are able to accept the second electron from cytochrome b_5. Cytochrome b_5 has also been shown to exert a differential stimulatory action, dependent upon the form of cytochrome P450 and substrate metabolised (Shimada et al., 2005; Pandey and Miller, 2005), (for review see Schenkman and Jansson, 2003; Miller, 2005).

In mitochondrial steroid hydroxylases and in the camphor hydroxylating bacterial P450 (CYP101) system a charge–pair interaction mechanism has been demonstrated by chemical modification, site-directed mutagenesis studies and structural data of electron transfer complexes (for review cf. Grinberg et al., 2000). Like microsomal reductase, the mitochondrial ferredoxin has also to deliver electrons to different cytochromes P450. From the available data, a shuttle model is favoured, where the oxidised ferredoxin interacts first with the ferredoxin reductase to undergo reduction with the formation of a Fe^{3+}/Fe^{2+} iron sulphur cluster. It dissociates from the reductase and then interacts with the respective cytochrome P450, where it delivers this electron before returning to the reductase and transferring the second electron to the P450.

The mechanism of electron transfer between the components of the different cytochrome P450 systems, one of the fundamental problems in life sciences, is not well understood yet. Although it could be conclusively shown that post-translational modifications can regulate these electron transfer reactions at least in some cases (Bureik et al., 2005), we are only at the very beginning of this field of research as well. Since electron transfer to the P450 in some cases seems to be low and rate limiting in P450 catalysis (Guengerich, 2002), engineering of this step could, however, lead to significantly improved biocatalysts.

4. Biotechnological applicability of cytochromes P450

Since cytochromes P450 catalyse valuable reactions on a vast variety of substrates they have a great biotechnological impact. Despite their impressive synthetic potential these enzymes have, however, so far only enjoyed very limited biotechnological use

due to their complexity, instability and, in most cases, low catalytic activity. Below, examples for successful application of cytochromes P450 in industrial processes will be provided, as well as examples for interesting *potential* applications of these enzymes as whole-cell or enzyme biocatalysts. Unfortunately, no data (besides with transgenic plants) on the scale of production, total turnover numbers of employed catalysts, productivity of fermentation, product costs and comparison with competing chemical processes have been published so far. This is due to the fact that we are still at the very beginning of industrial application of these enzymes. Nevertheless, there is promising progress in preparing these complex systems for biotechnological application.

4.1. Transgenic plants

The first example of a product on the market where P450 has been used for its production are transgenic plants. Since traditional hybridization failed to produce blue roses, there has been a vital interest in producing those plants during the past decade using genetic engineering techniques. Scientists from Florigene/Australia and from Suntory/Japan finally succeeded in producing delphinidin, responsible for the blue color, in roses by introducing the respective P450 gene (*CYP75A*) involved in this biosynthesis from blue pansy, expression of the dihydroflavonol reductase from petunia and suppression of the rose dihydroflavonol reductase (Holton et al., 1993; Ogata et al., 2005). However, so far the color is still far from being "pansy blue" which is due to the pH in the vacuoles. The method has, however, successfully been applied for the production of carnations (Fukui et al., 2003) with new and very pretty colors (from light to deep purple), which already nowadays have very high selling rates. When taking into account that the global market of cut flowers is in the range of about $27 billion per year, this seems to be of economical impact.

4.2. Microorganisms as potential biocatalysts

4.2.1. The synthetic power of native microorganisms

Screening of microorganisms for special biosynthetic functions revealed cells containing various P450 systems involved in the biosynthesis of biotechnologically interesting compounds. However, as mentioned above, the potential of microbial P450s in biotechnology has so far scarcely been used.

One of the first microorganisms with potential biotechnological application were alkane-hydroxylating yeast strains (Schunck et al., 1978; Honeck et al., 1985; Sanglard et al., 1987; Zimmer et al., 1996; Ohkuma et al., 1995). More recently, transformation of fatty acids by *Candida tropicalis* and identification of the reaction steps catalysed by different isoforms of this microorganism as a basis for the design of this yeast have been performed (Eschenfeldt et al., 2003). Another *Candida* strain, *Candida bombicola*, immobilized on patterned lipid films was used for the transformation of arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE) (Phadtare et al., 2003). And very recently, application of *Yarrowia lipolytica* for the oxidative degradation pathways of alkanes and triglycerides has been given (for an overview see Fickers et al., 2005).

In addition, various organisms have been checked during the past few years concerning their ability to produce interesting compounds using endogenous P450s. Screening 1800 bacterial strains with alkane-hydroxylating abilities, a bacterium, *Mycobacterium* sp. strain HXN-1500 was identified being able to specifically hydroxylate 1-limonene in the 7-position to produce enantiopure (−)-perillyl alcohol, which is a promising compound with anti-carcinogenic properties (van Beilen and Funhoff, 2005). Bioconversion of compactin into pravastatin, an anti-hypercholesterolemic drug as well as the production of antibiotics by *Streptomyces* sp. has been described, as has been the production of epothilone, an anti-cancer agent, by *Myxobacteria* (Lamb et al., 2003; Beyer et al., 1999; Tang et al., 2000; Molnar et al., 2000; Gerth et al., 2003; Park et al., 2003).

It is clear that biological diversity offers a broad field of new applications for P450-catalysed biotransformation. This potential is so far not used to a noticeable degree. However, it offers the opportunity to find many new and highly interesting organisms for biotechnological application with P450-catalysed product formation.

4.2.2. Microorganisms as hosts for P450 expression

To apply cytochrome P450s in biotechnology either whole-cell systems expressing the P450 isoforms of

interest have to be developed or self-sufficient systems avoiding NAD(P)H regeneration have to be used. In general, whole cells are more popular than (partly) isolated enzymes in biotechnological processes (Straathof et al., 2002).

The most widely studied P450 for biotechnological application nowadays is CYP102 from *Bacillus megaterium* (see also below) due to its potential to hydroxylate a variety of alkanes, fatty acids as well as aromatic compounds and due to its high activity (Urlacher et al., 2004 and references cited therein). Applying CYP102 in a whole-cell system after its expression in *Escherichia coli* does not require exogenous addition of the costly cofactor NADPH and appears to be a useful alternative to the enzymatic approach for the bioconversion of fatty acids (Schneider et al., 1998).

Four genes encoding sequential steps in the biosynthesis of the spearmint monoterpene ketone (−)-carvone from its isoprenoid precursors have been expressed in *E. coli* and lead to the production of nearly 5 mg/l of the pathway intermediate (−)-limonene (Carter et al., 2003). Further studies are necessary to overcome excretion of this intermediate and to produce (−)-carvone. Sakaki et al. developed an expression system which can be used to construct a bioreactor for the production of 1α,25-dihydroxyvitamin D3 and vitamin D analogs (Sakaki et al., 1999). His group was able to express the renal CYP27B1 and CYP24 in *E. coli* together with their electron transfer partners, adrenodoxin reductase and adrenodoxin to allow the vitamin D hydroxylases to work in this heterologous system. A similar system for the production of steroids has been described very recently by our group (Hannemann et al., 2006). CYP106A2 from *Bacillus megaterium* has been expressed in *E. coli* together with adrenodoxin and adrenodoxin reductase to allow the production of hydroxylated steroid derivatives of 11-deoxycortisol or progesterone. This system can also be used as a screening system for molecular evolution of the steroid hydroxylase. Thus, production of highly active steroid hydroxylases with differing regio- and stereoselectivity of substrate hydroxylation will become available using this system.

Expression of cytochromes P450 has also been reported in various yeast strains. Already the group of Ohkawa (at that time at the Sumitomo Chemical Co., Japan) succeeded in expressing a membrane-bound P450 together with the corresponding electron transfer proteins in *Saccharomyces cerevisiae* (Ohkawa et al., 1990; Shibata et al., 1990; Akiyoshi-Shibata et al., 1991; Sakaki et al., 1992). Expression of various microsomal P450s involved in the biotransformation of drugs or the degradation of xenobiotics in *Saccharomyces cerevisiae, Pichia pastoris* or *Yarrowia lipolytica* has been reported meanwhile and can be used to produce biotransformation intermediates necessary for the characterisation of new potential drugs (Andersen, 2000; Nthangeni et al., 2004; Fickers et al., 2005). The conversion of 11-deoxycortisol into cortisol (hydrocortisone) can be followed using recombinant *Schizosaccharomyces pombe* strains that express human CYP11B1 (Bureik et al., 2002; Dragan et al., 2005). The production level is considerably higher than that of CYP11B1 expressed in *Saccharomyces cerevisiae* (Dumas et al., 1996). Interestingly, coexpression of adrenodoxin, which is essential to obtain cortisol in the baker's yeast system (Dumas et al., 1996), was not necessary in fission yeast, since an endogenous iron–sulphur protein, etp1, was able to substitute adrenodoxin (Bureik et al., 2002). The ferredoxin domain of this protein, moreover, was demonstrated to be very similar to the mammalian adrenodoxin, the natural electron transfer partner of CYP11B1 (Schiffler et al., 2004a,b).

An excellent example that P450s can indeed be used in biotechnological processes is the construction of engineered yeast for the biosynthesis of hydrocortisone (Duport et al., 1998; Szczebara et al., 2003). For this purpose the catalytic power of four P450s has been used in a whole-cell system. An artificial and fully self-sufficient biosynthetic pathway involving 13 engineered genes was assembled and expressed in a single yeast strain. As a result, hydrocortisone can be produced from a simple carbon source. This process is on the border of being introduced into the production process by Sanofi-Aventis Co. It has to be mentioned that the development of this process needed more than a decade but provided the company with unique experiences in the construction of complicated pathways into yeast and with a set of building blocks, which now can also be used for other, similar biotechnological processes. It also shows that application of P450s as biocatalysts (although admittedly being complicated and needing broad interdisciplinary experiences, especially deep understanding of this class of enzymes) is

possible if the industry is willing to follow a long-term goal.

4.3. Engineering of P450 substrate specificity

4.3.1. Engineering using site-directed mutagenesis

Bacterial P450 systems normally exhibit higher catalytic rates than eukaryotic P450s and are easy to handle in the laboratory due to their solubility and high expression level in heterologous hosts. These properties also make bacterial P450s ideal targets for protein engineering. Most work has been focussed on the two proteins CYP101 ($P450_{cam}$) and CYP102 (P450 BM-3), and both have been engineered to accept a wide variety of substrates. CYP101, from *Pseudomonas putida*, requires two additional electron transfer proteins, putidaredoxin, a ferredoxin of the [2Fe–2S] type, and putidaredoxin reductase, a FAD-containing enzyme, for catalysis, while CYP102 from *Bacillus megaterium* contains its heme and reductase domains in a single polypeptide chain (Fig. 2). Both proteins show hydroxylation rates in the range of thousands min^{-1}. These two proteins were the first two members of the P450 superfamily of which crystal structures became available (Poulos et al., 1985; Ravichandran et al., 1993) providing research groups with some insight into their mechanisms and ideas for their rational engineering. Additional crystal structures of these enzymes bound to different substrates and containing mutations (see: Brookhaven data base http://www.rcsb.org/pdb/) have become available and have also contributed to our knowledge about this important group of enzymes. Most work with these systems has focussed on altering the specificity and regioselectivity of hydroxylation reactions catalysed by these proteins.

First attempts to engineer a P450 for potential use in bioremediation have been performed with CYP101, which naturally hydroxylates camphor in the 5-exo position. On the basis of the known three-dimensional structure of substrate-bound CYP101 (Poulos et al., 1987) substitution mutants Thr101Met, Thr185Phe, and Val247Met have been produced to re-engineer the stereochemistry and coupling of ethylbenzene hydroxy-lation. The reaction with the wild-type produces one regioisomer, 1-phenylethanol, with only 5% coupling (e.g. 95% of reducing equivalents are governed into the formation of hydrogen peroxide or water) and a ratio of 73:27 for the *R* and *S* enantiomers, respectively. The triple mutant hydroxylates ethylbenzene resulting in a *R*:*S* ratio of 87:13 and a coupling of reducing equivalents to product of 13% (Loida and Sligar, 1993a,b). Various mutants of Tyr96 (Tyr96Ala, Tyr96Gly), which in the original enzyme is hydrogen-bonded to the natural substrate of CYP101, camphor, create active sites that improve the oxidation of phenyl derivatives, e.g. diphenylmethane, diphenylamine and 1,1-diphenylethylene, which are not converted by the wild-type enzyme (Bell et al., 1997). Moreover, the ability of CYP101 to perform styrene oxidation was improved 25-fold using mutant Tyr87Phe with an increase in the coupling efficiency from 7 to 32% (Nickerson et al., 1997). Mutations in Phe87 and Tyr96 also greatly enhanced the activity of CYP101 for the oxidation of the polycyclic aromatic hydrocarbons phenanthrene, fluoranthene, pyrene and benzo[a]pyrene as well as for polychlorinated benzenes (Harford-Cross et al., 2000; Jones et al., 2001). When the space available at the top of the active site was decreased with mutant Phe87Trp, this mutation obviously forced the substrate to be bound closer to the heme resulting in a 10-fold increase in activity with most of the polychlorinated substrates. The coupling rate was increased to >90% by bringing in an additional mutation, Phe98Trp, although the NADH-dependent reaction decreased in those mutants (Jones et al., 2001). Mutants Phe87Trp and Tyr96Trp were also shown to improve regioselectivity of 2-ethylhexanol oxidation. The mutant Thr185Phe improved coupling of NADH supported product formation and Leu244Ala altered the stereoselectivity of ethylhexanoic acid production (French et al., 2002). A definitive demonstration of the flexibility of the P450 platform was provided by Wong and coworkers (Bell et al., 2002) with the engineering of CYP101 to hydroxylate short-chained alkanes. In this study the volume of the active site above the heme was decreased by introducing bulky, hydrophobic residues, with the mutant F87W/Y96F/T101L/V247L oxidizing butane with a rate of 750/min that was 95% coupled to NADH oxdiation. The rate of propane hydroxylation in this study was 110/min with 32% NADH coupling. Very recently CYP101 was further designed to convert ethane to ethanol (Xu et al., 2005). Some of the mutants designed by the group of Wong were also used for other biotransformations. The crystal structures of the mutant F87W/Y96F/V247L with 1,3,5-triclorobenzene or (+)-alpha-pinene bound

provided a basis for further engineering of CYP101 for increased activity in the oxidation of the inert pentachlorobenzene and hexachlorobenzene and increased selectivity of (+)-alpha-pinene oxidation (Chen et al., 2002; Bell et al., 1997; Bell et al., 2003).

Wild-type CYP102, the fastest known P450, primarily hydroxylates fatty acids with chain length between C12 and C18. In crystal structures of CYP102 a hydrophobic channel between the surface of the protein and the proximal side of the heme is evident. The hydrophobic residues in this channel presumably contact the substrate during catalysis. In particular, the amino acid residue F87 has been shown to affect both the substrate specificity and regioselectivity of fatty acid hydroxylation (Graham-Lorence et al., 1997). Replacement of F87 with valine converted the enzyme into a regio- and stereoselective arachidonic acid epoxygenase. Mutants of this residue were used as the starting point for both the creation of new site-directed mutants with specificities towards non-natural substrates such as alkanes and arene compounds and for the initiation of directed evolution experiments to evolve this P450 for biotechnological application (see below). Mutant F87V also showed increased activity towards a variety of aromatic and phenolic substrates (Sulistyaningdyah et al., 2005).

The mutant F87V/L188Q/A74G was designed by saturation mutagenesis of multiple sites that were chosen based on crystallographic data. This approach, termed "rational evolution" was used with the assistance of a *p*-nitrophenol based screen. The mutant was shown to oxidise indole and can also hydroxylate alkanes, cycloalkanes, arenes and heteroarenes (Appel et al., 2001). It was later shown that this mutant can also hydroxylate polycyclic aromatic hydrocarbons such as naphthalene, fluorene, acenaphtene, acenaphtylene and 9-methylanthracene. However, coupling efficiencies for this mutant were very low. Moreover, this mutant is able to metabolise polychlorinated dibenzo-*p*-dioxins (Sulistyaningdyah et al., 2004; Sulistyaningdyah et al., 2005). Carmichael and Wong (2001) were also successful in obtaining activity towards polycyclic aromatic hydrocarbons using site-directed mutants of CYP102. The mutant R47L/Y51F/A264G from this study had its highest product formation rates towards phenanthrene and fluoranthrene while mutant R47L/Y51F/F87A/A264G was most active towards pyrene. This study also observed very low NADPH coupling efficiencies.

Engineering of other P450s besides CYP101 and CYP102 for biotechnological use is compromised by the observation that many of them possess only low activities. This is, however, counterbalanced by the fact that they open up completely different products to be synthesized which often are extremely difficult to produce by chemical means, such as steroid molecules with hydroxyl groups in specific positions. Thus, the structural basis for the regio- and stereoselectivity of steroid hormone hydroxylation has been studied using residue swapping of human CYP11B1 and CYP11B2 and has been attributed to the putative I-helix (Bottner and Bernhardt, 1996; Bottner et al., 1996, 1998). Additional residues responsible for the selectivity of steroid hydroxylation have been found also in the N-terminal part of the sequence (Bechtel et al., 2002). This knowledge, together with computer modeling of the protein, has been applied to design a bacterial steroid hydroxylase, CYP106A2, with changed hydroxylation selectivity (Lisurek et al., in preparation).

4.3.2. Engineering using laboratory evolution

CYP102 is particularly well suited for directed evolution experiments since it is highly expressible in *E. coli*, soluble (i.e. not membrane bound), stable, fast (rates up to 3000 min^{-1}), and self-sufficient (i.e. the reductase, electron transfer, and hydroxylase components are included in a single protein). Using the methods of directed (molecular, laboratory) evolution, a library of randomly mutated CYP102 proteins is generated and screened for new or improved activities. Mutants with improved activities are isolated and then used to generate new libraries using again random mutagenesis or recombination technologies. This iterative cycle continues until the desired mutant or mutants is/are obtained (for review cf. Arnold and Georgiou, 2003; Arnold et al., 2001). The mutations accumulated over several rounds of directed evolution often appear in unpredictable places in the protein (i.e. not in the active site), illustrating the limitations of rational approaches to protein engineering.

Schwaneberg et al. (Schwaneberg et al., 2001), using a product-based screen for detecting mutants with increased fatty acid hydroxylation activity with a "surrogate" substrate (Schwaneberg et al., 1999) were able to generate mutants that oxidized *n*-octane up to five

times faster than the wild-type enzyme. By additionally employing a cofactor dependent screen using short alkanes as substrates a mutant was generated that readily oxidized not only octane at a higher rate than any known alkane hydroxylase but also propane (Peters et al., 2003). This enzyme was also more active towards its natural substrates. None of the eleven amino acid substitutions were homologous to the mutations (Bell et al., 2002) introduced into CYP101 to achieve butane and propane oxidation. Interestingly, mutant 139-3, exhibiting high activity towards a variety of fatty acids and alkanes is also active on benzene, styrene, cyclohexene, 1-hexene, and propylene. It has, however, to be mentioned that NADPH consumption is only partially coupled to product formation (14–79%) leading to the formation of reactive oxygen species (Farinas et al., 2004). While the enzyme exhibited high rates of substrate hydroxylation, it was not regioselective and produced a variety of subterminal alcohols. Using a combination of directed evolution and site-directed mutagenesis, the mutant was further engineered to hydroxylate alkanes regio- and even enantioselectively. CYP102 variant 9-10A-A328V hydroxylates octane at the 2-position to form *S*-2-octanol (40% ee). Another variant, 1–12G, also hydroxylates alkanes larger than hexane primarily at the 2-position, but forms *R*-2-alcohols (40–55% ee). Both biocatalysts are highly active (rates up to 400 min^{-1}) and support thousands of product turnovers (Peters et al., 2003). Moreover, mutants 139-3 and 1-12G, as well as mutant B, were able to perform stereoselective hydroxylation of an achiral cyclopentanecarboxylic acid derivative being of importance as intermediate for the development of drugs against HIV (Munzer et al., 2005). Very recently, using a combination of saturation mutagenesis and recombination, CYP102 mutants were obtained which were able to produce ethanol from ethane although still at rather low activity (Meinhold et al., 2005).

The activity of CYP102 and its mutants have also been tested with a variety of other substrates of potential biotechnological importance. Thus, biotransformation of β-ionone has been described using mutant R47L/Y51F/F87V. This triple mutant exhibited moderate enantioselectivity, forming (*R*)-4-hydroxy-β-ionone with an optical purity of 39% (Urlacher et al., 2005).

Since CYP102 catalyses the hydroxylation and/or epoxidation of broad ranges of substrates, including very hydrophobic ones its ability to be active in organic solvents has also been improved using directed evolution (Seng Wong et al., 2004).

As in the case of engineering using site-directed mutagenesis, there are few examples for application of the methods of laboratory evolution to P450s other than CYP101 and CYP102. An expression and screening system which can be applied for laboratory evolution of the soluble steroid hydroxylase CYP106A2 is ready for use and will certainly lead to steroid molecules, e.g. progesterone derivatives, with hydroxyl groups in different positions (Hannemann et al., 2006). A successful example of laboratory evolution on mammalian P450s has been published by (Nakamura et al., 2001), who applied random mutagenesis to the substrate recognition sequences (SRS) of the indole-hydroxylating CYP2A6 and identified mutants that form primarily indigo from indole. *E. coli* containing randomly mutated CYP2A6 were selected based upon their ability to form the blue dye, resulting in the identification of several mutants that form different mixtures of indigo-containing indole oxidation products. Indigo formation has also been reported in mutants of the bacterial P450 CYP102 (Li et al., 2000, 2005).Whether this pathway can be used to improve blue color formation in roses needs further investigation.

Taken together, these results clearly demonstrate the potential of cytochromes P450 for the production of a variety of biotechnologically interesting compounds. The main shortcoming is, however, that the system is not easy to use due to its dependence on the cofactor NADPH. Therefore, attempts have been undertaken to replace the cofactor by using the peroxygenase activities inherent to many P450 systems (see below).

4.3.3. Engineering using chimeragenesis

Chimeragenesis has previously been successfully performed using P450s of the same subfamily (for review, see Domanski and Halpert, 2001), which show a high sequence homology (due to definition, more than 55%) to change substrate selectivity and activity and to identify individual amino acid residues involved therein. New activities have been observed in some of these chimeric mutants (Straub et al., 1994). Chimeragenesis has also been applied to P450s that share only low sequence homology (around 20%) in order to solubilize membrane-bound P450s. Already in 1998 Shimoji et al. (Shimoji et al., 1998) applied rational

chimeragenesis to solubilize a membrane-bound protein. These authors were able to construct a soluble chimera from ∼50% bacterial CYP101 and ∼50% mammalian membrane-bound CYP2C9, which was functionally active in 4-chlorotoluene oxidation.

Nature uses a similar principle, recombination of genes, to produce a variety of chimeric genes, which might be advantageous for the development of an organism. Thus, various recombination methods such as DNA shuffling (Stemmer, 1994), StEP recombination (Zhao et al., 1998) and others (Miyazaki and Arnold, 2004) have been applied also for laboratory evolution. Recombination of homologous proteins generates variants in which every amino acid substitution has already proven to be successful in one of the parents, making recombination more efficient than random mutagenesis for generating mutants with new properties. The creation and characterisation of a highly chimeric library has been described by (Abecassis et al., 2000, 2001), who produced a library of CYP1A1 and CYP1A2 chimeras using a combination of PCR-based and in vivo recombination in yeast. However, genes generally need to exhibit very high levels of sequence identity (normally of 70% or higher) in order to be successfully recombined and crossovers are biased towards regions of high sequence identity (Ness et al., 2000; Lutz et al., 2001; Voigt et al., 2002). P450s generally share very low sequence identity, often <20% and thus traditional recombination methods are not applicable. In addition, these crossover points do not necessarily represent the optimal crossover locations that lead to proper folded and functional proteins, since so far no algorithms were available to predict regions where chimeragenesis would be not harmful to the stability of the protein. Thus, in all cases, where P450s with low homology were used a considerable amount of the constructed chimeras as well as large parts of the library were giving unstable mutants.

The approach of Arnold's group reported very recently (Otey et al., 2004, for comments see also Bernhardt, 2004a,b) overcomes this shortcoming by applying a powerful structure-based algorithm, SCHEMA (Voigt et al., 2002; Meyer et al., 2003), which identifies fragments of proteins that can be recombined to minimize disruptive interactions that would prevent protein folding. The authors used SCHEMA to design chimeras of the closely related model proteins CYP102A1 and CYP102A2, sharing 63% amino acid sequence identity. Fourteen of the 17 constructed hybrid proteins were folded as determined by CO difference spectroscopy. Out of them, 13 gave rise to a peak at 450 nm, whereas only one lead to the inactive P420. This is an impressing high fraction of structurally folded proteins. Interestingly, half of the chimeras had altered substrate activities, while three mutants acquired activities towards a new substrate not hydroxylated by either parent. The method proposed by the authors should be generally applicable for creating diverse libraries of chimeric P450s and opens new and exciting opportunities for engineering P450s.

4.3.4. Engineering improved redox partner interaction

The potential of improving redox partner interaction and electron transfer for biotechnological application of P450 systems has been so far widely underestimated. As external monooxygenases, most cytochromes P450 have to interact with partner proteins to receive electrons for oxygen activation and substrate conversion. Only a few P450s do not require additional proteins for catalysis such as CYP102 with its hydroxylase and reductase domains fused into a single polypeptide chain. In many P450s, the transfer of the second electron from the reductase to the hydroxylase component is rate limiting for most substrates. Thus, improving this interaction offers an efficient tool to enhance product formation in biocatalytic processes. To enhance electron transfer rates, understanding of the protein–protein interaction and electron transfer in these systems is necessary (for review see: Bernhardt, 1996; Grinberg et al., 2000; Guengerich, 2002). Elucidation of key residues involved in protein–protein interaction and electron transfer typically makes use of mutants that in one way or another destroy activity or binding. These changes must be treated very carefully, since effects on parameters other than a direct involvement in the function studied could account for the observed changes. It is comparatively much more difficult to obtain mutants in which these properties are improved. A mutant of adrenodoxin (Adx) with improved electron donor function in mitochondrial systems has been obtained. This mutant, Adx (4-108), was 4-fold better for CYP11B1-dependent substrate hydroxylation than wild-type Adx (Uhlmann et al., 1994). The efficiency of substrate conversion (k_{cat}/K_m) is enhanced 22-fold for CYP11B1 and 3-fold for

CYP11A1. Introducing a tryptophan into position 112 of Adx (S112W) led to increased binding affinity and electron transfer rate to CYP11A1 (Schiffler et al., 2001; Schiffler and Bernhardt, 2003). Additionally, the k_{cat} value of this mutant with CYP11A1 is significantly enhanced as is the efficiency of substrate conversion (k_{cat}/K_m), which is 75-fold higher than that of the wild-type. An even further increase (100-fold) in efficiency was obtained by the double mutant S112W/Y82F.

Very recently enhanced electron transfer and substrate conversion was also observed for a mutant of the thermophilic CYP119 (D77R) when reconstituted with the heterologous redox partner putidaredoxin to create an efficient thermostable fatty acid hydroxylase (Koo et al., 2002).

Taken together, the methods of laboratory evolution will surely lead to further improvement of redox partner interaction and increased substrate conversion.

4.4. Peroxide-driven catalysis

Application of most P450s as oxidation catalysts is limited due to the dependence on the expensive cofactor NAD(P)H. An inefficient peroxide shunt pathway, in which oxygen pre-reduced by two electrons to a peroxide is used for catalysis in place of dioxygen and NAD(P)H (see Chapter 2), exists in most P450s and allows them to function without the additional cofactor and electron transport proteins. Directed evolution was used to improve peroxide-driven catalysis of CYP101 and CYP102. By random mutagenesis and subsequent DNA shuffling of CYP101 (Joo et al., 1999a,b) were able to increase its H_2O_2-driven hydroxylation of naphthalene 20-fold. Cirino and Arnold (2002) characterised hydrogen peroxide-driven catalysis with the heme domain of CYP102 and its mutant F87A and found that mutation F87A significantly enhances peroxygenase activity towards medium-chain fatty acids while shifting regioselectivity away from the terminal position. However, catalytic rates using hydrogen peroxide were still orders of magnitude slower than using NADPH as a cofactor. Using directed evolution, (Cirino and Arnold, 2003) obtained a mutant CYP102 heme domain supporting the H_2O_2-driven hydroxylation of fatty acids and epoxidation of styrene at catalytic rates and total turnover numbers improved by an order of magnitude relative to the F87A mutant although a significant decrease in its thermostability was observed. None of the amino acid changes in this mutant (relative to wild-type CYP102) were located in the substrate-binding pocket. Moreover, directed evolution also allowed engineering of this enzyme to efficiently hydroxylate alkanes (Glieder et al., 2002). The crucial problem in applying the hydroperoxide shunt for self-sufficient substrate conversion of cytochromes P450 seems, however, to lie in the action of the hydroperoxides onto the active site leading to time-dependent destruction of the heme group. Attempts to increase the affinity for the peroxide, thus lowering the applied amount of the agent and protection of the heme by changing the surrounding of the active site might help to construct active mutants with reasonable stabilities. Thus, in a report on the thermostabilization of a CYP102 (Salazar et al., 2003) were able to restore the loss in thermostability while maintaining its peroxygenase activity using directed evolution.

5. Outlook

The diverse chemistry catalysed by members of the cytochrome P450 family opens up new potentials for practical application of these protein systems. Besides this, the high regio- and stereoselectivity of hydroxylation reactions catalysed by the members of the P450 gene family are of special interest.

So far, industrial application is, however, focused on only very few cases. The most prominent example is the production of transgenic plants such as carnations and blue roses. The other example is the production of hydrocortisone using a designed strain of Baker's yeast expressing a total of four P450s and engineering 13 proteins (see above). Both projects needed developmental research for more than 10 years but now seem to be of high commercial value. It is worth to point out that these processes are of future value, since they are built of blocks, which can be used also for the production of similar products (other kinds of flowers, other steroids). Moreover, application of the designer yeast for hydrocortisone production represents a sustainable process, which is not only state-of-the-art but also more environment-friendly. These examples show that although biotechnological application of P450s is feasible, promising and obviously can lead to high profits, it needs a long time and excellent knowledge of the corresponding P450 systems to be applied and

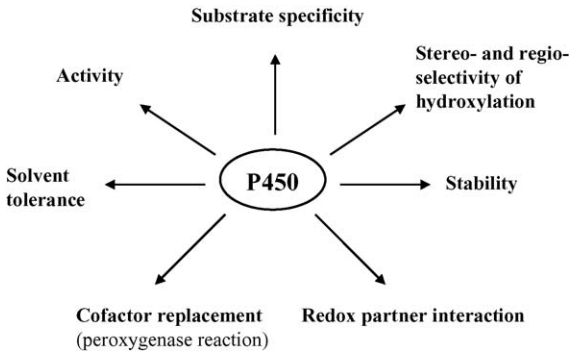

Fig. 3. Improvement of cytochrome P450 system properties for potential biotechnological application using site-directed mutagenesis, laboratory evolution and chimeragenesis.

the hosts to develop the industrial processes due to the complexity of these enzyme systems.

Recently, several approaches to prepare these complex systems for further biotechnological application have been undertaken (Fig. 3), but it seems to be still too early for these improved P450 systems to be applied in the industry, although it is possible to change the regio- and stereoselectivity of hydroxylation, to improve activity, stability and coupling of the reaction significantly and to enable P450 systems to work in organic solvents.

To reduce this complexity of the P450 systems attempts have been undertaken to construct self-sufficient systems, which, instead of using the costly cofactor NAD(P)H are able to catalyse substrate conversion using peroxides. Indeed, with CYP102, the P450 with the highest activity described so far, a set of different reactions of potential biotechnological use have been catalysed using peroxide-driven catalysis. Moreover, site-directed mutagenesis and molecular evolution were successfully applied to improve the activity of this system and to change the substrate specificity. The shortcoming of this system is the ongoing destruction of the active site heme in the presence of peroxides, a problem which has to be solved before this system will become applicable for biocatalysis.

Site-directed mutagenesis, directed evolution and chimeragenesis have been used and seem to be of extraordinary success to improve the activities of various P450s, especially CYP101 and CYP102, and to create new mutants with designed reaction specificities. This approach will clearly challenge traditional chemical approaches for the synthesis of bulk as well as high-value chemicals. Although quite different products have been obtained using CYP101 and CYP102 as catalysts, more complicated products might be more efficiently produced using other P450 isoenzymes. Thus, steroid hydroxylation is performed with high stereo- and regioselectivity by CYP106A2 and the activity of this reaction could be improved by laboratory evolution. Very recently, systems have been established to create mutants of this protein with changed selectivities of hydroxylation so that steroid molecules with hydroxyl residues in different positions of the steroid molecule should become available in the near future using biotransformation. To further increase the activity of P450s, site-directed mutagenesis has been performed to improve their redox partner interaction. It was shown that a deep knowledge of protein–protein interactions and electron transfer of the respective systems can lead to impressing improvements (up to 100-fold) of their catalytic efficiencies.

Moreover, site-directed mutagenesis has very successfully been applied to stabilize and/or solubilize proteins of different P450 systems, especially membrane-bound microsomal P450s, as a prerequisite of crystallisation. Thus, microsomal CYP2C5 has been engineered as a soluble, monomeric enzyme, being able to crystallize and diffract (Cosme and Johnson, 2000) providing us with the first 3D structure of an originally membrane-bound P450 (Williams et al., 2000). Other microsomal P450s have been solubilized using the same method (see Brookhaven data base http://www.rcsb.org/pdb/) This will certainly also enforce their application for biotechnological purposes.

Finally, the diversity of P450s becoming available through the sequencing of genomes of a variety of organisms opens up new sources for application. Cytochromes P450 are being found in all kingdoms, from eubacteria, archaebacteria, fungi, plants, fish, insects to vertebrates. More than 250 different forms have been found in the plant *Arabidopsis thaliana* and even more than 1000 isoforms are being expected in wheat. It was shown that plant P450s play a role in the metabolism of a variety of secondary metabolites, in plant–insect interaction, herbicide metabolism and other vital functions (Werck-Reichhart and Feyereisen, 2000). Moreover, the increasing number of discovered

plant P450s revealed their potential for engineering herbicide tolerance, biosafening, bioremediation and green chemistry (Werck-Reichhart et al., 2000). Similar varieties of P450 isoforms have been found in bacteria and fungi. Thus, a set of different reactions with new products will become available by studying and applying these new P450 systems. Two examples for model organisms with biotechnologically interesting product formation are *Streptomyces* and *Myxobacteria*. Eighteen P450 isoforms have been identified in *S. coelicolor* (Lamb et al., 2002) and a similar number can be expected in *Sorangium cellulosum*. It is well known that *Streptomyces* species are involved in the production of about two-thirds of naturally occurring antibiotics and a wide array of other secondary metabolites. Taking into account the necessity to develop new antibiotics for overcoming problems of resistance, the characterisation of *Streptomyces* P450s that might be involved in the biosynthesis of antibiotics and other secondary products is of high biotechnological relevance (Lamb et al., 2003; Podust et al., 2004; Zhao et al., 2005). When considering the products formed by *Myxobacteria* it is impressive that many secondary metabolites are being produced by these bacteria, e.g. myxovirescin, soraphen and epothilone (Beyer et al., 1999; Gerth et al., 2003). The epothilones were shown to possess anticancer activity. Thus, characterisation of the "CYPom" of these bacteria will also lead to the identification of new P450s playing an important role in the production of pharmaceutically important compounds. New fields of application of the potential of P450s in biotechnology will open. Thus, their involvement in the natural production of fruit flavors, aroma compounds as well as fragrances will induce new applications (Aharoni et al., 2004; Sowden et al., 2005). Taken together, due to the diversity of catalysed reactions it can be anticipated that P450s will create a broad field for future applications. The potential of this class of enzymes for biotechnological application thus seems to be tremendous.

Summarising, we seem to be at the threshold of a new century, where the diversity of P450-catalysed reactions will become available and applicable for biotechnological use due to the thousands of isoforms of this enzyme converting thousands of different substrates to new products and due to the possibilities of genetic engineering techniques allowing to express these proteins in different hosts and to use various mutagenesis methods to produce an even higher amount of isoenzymes with higher activities as well as new and changed selectivities.

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