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**Chimeric P450 Enzymes: activity of artificial redox fusions driven by different reductases for biotechnological applications**

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Running Title:

Artificial fusions of CYP haem domains with different reductase proteins

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**Synopsis**

This review covers the current state of knowledge regarding artificial fusion constructs of cytochrome P450 enzymes where the activity of the catalytic haem is driven by reductases of different origins.

**Key words**

Chimera, CYP, flavodoxin, fusion, protein engineering, reductase

**Abstract.**

Cytochromes P450 form a vast family of haem-thiolate proteins which act as monooxygenases by activating molecular oxygen resulting in the insertion of one atom into an organic substrate with the concomitant reduction of the other to water. The reducing equivalents are usually supplied by NADH or NADPH and are transferred in two consecutive steps via the redox partner(s). These include reductases containing FMN and/or FAD and/or Fe-S clusters in different combinations depending on the P450 system. These enzymes catalyse extremely diverse reactions including regio- and stereospecific oxidations of a large range of substrates in addition to many drugs and xenobiotics, as well as biosynthesis of physiologically important compounds such as various steroids, vitamins and lipids. Because of their ability in catalysing such vast range of reactions they have become the focus of biotechnological interest, but their dependence on the reductase partner has remained one of the challenging limitations for full exploration of their synthetic potential. In order to address the latter limitation many researchers have reconstituted functional P450 enzymes by fusion to different reductase proteins and this review will cover their findings.

**1. Introduction**

The cytochrome P450 enzymes (CYPs or P450s) are a family of haem-thiolate proteins that currently contains over 7000 members in species ranging from bacteria through to plants and mammals [1]. These enzymes were first recognized by Martin Klingenberg [2] who was studying the spectrophotometric properties of pigments present in the microsomal fraction from rat livers. Upon addition of the reducing agent sodium dithionite and bubbling carbon monooxide, a unique spectral absorbance band with a maximum at 450 nm was observed hence the name p450 i.e. “pigment” 450. Several years later Omura and Sato not only described it as a new cytochrome and called it “P-450” [3] but also determined an extinction coefficient increment for it in the CO-difference spectrum, a value essential for determination of P-450 content [4].

These enzymes are generally involved in the metabolism of xenobiotics, in particular in humans they are crucial in hepatic phase-1 drug metabolism. In addition, they are attractive as biocatalysts due to their ability to catalyse regio- and stereo-specific oxidative reactions of unactivated C-H bonds. Their versatility in the recognition of different substrates, all of technological relevance either for the development of new drugs or for the detection or degradation of environmentally relevant molecules, has led to an enormous amount of protein engineering work aimed at improving or expanding their catalytic performance for biotechnological applications [5-7].

The reaction catalysed by the P450 enzymes involves the activation of molecular oxygen which is usually through sequential electron transfer from NAD(P)H to the P450 haem via a redox partner(s). This inherent dependence on the reductase and the use of NAD(P)H to initiate the electron transfer has limited the development of these enzymes as biocatalysts [8]. In order to bypass the latter limitations, alternative approaches for delivering electrons have been developed, that include artificial fusions or chimeric P450 proteins [9-13], electrochemical reductions [14] as well as light-activated approaches [15, 16].

One of the most effective modes of bypassing the inclusion of external reductases is to construct self-sufficient P450 enzymes by fusing the naturally separate proteins within the redox chain. Fusion of the domains/proteins can be brought about by simply engineering the peptidic regions between the C- and N-termini of the proteins or by specific disulfide bridges at sites of redox interaction or by using peptide linkers which are present in naturally fused P450 systems. There is also the possibility of shuffling and using different redox domains (“molecular Lego” [9, 10]) in order to improve electron transfer to ultimately enhance product formation in biocatalytic processes of these enzymes. The feasibility and potential of the latter approach has been largely underestimated but certainly has implications in the successful exploitation of P450 systems in biotechnological applications. In light of this, the present review covers the P450 fusions that have shown artificial redox chains for driving the catalysis.

**2. Redox chains of cytochrome P450 enzymes**

Cytochrome P450 enzymes act as monooxygenases and catalyse the insertion of one atom of oxygen into the organic substrate RH with the concomitant reduction of the other oxygen atom into water, using the reducing power of NADP(H) as shown below:

**RH + O2 + H+ + NADPH →ROH + H2O + NADP+**

In many P450 enzymes the mechanism involves two successive one-electron transfer steps where electrons originating from NADP(H) are transferred to the P450 haem centre *via* a redox partner. Many different types of reactions catalysed by P450s have been identified [17] including hydroxylation, dealkylation, heteroatom oxygenation and epoxidations, to name a few.

Recently, Bernhardt and colleagues compiled a classification of known and recently discovered cytochrome P450 enzymes based on the organization of their different domains leading to ten different classes [18] as summarised in Table 1. However, only four classes, which are the ones that have been mainly used in cytochrome P450 fusion constructs and relevant to this mini-review, will be discussed in more detail.

Class I (Figure 1A) are three component systems consisting of a flavin adenine dinucleotide (FAD) containing reductase that oxidises NADH to NAD+, an iron-sulphur protein (e.g. ferredoxin) that acts as an electron carrier and the cytochrome P450. Members of this class include mitochondrial or bacterial P450s.

The bacterial cytochromes P450 of this class are involved in the catabolism of compounds used as carbon source, the metabolism of fatty acids and the production of antibiotics or antifungals. The most studied bacterial P450 of this class is that of the P450cam (CYP101) from *Pseudomonas putida*, which catalyses the 5-exo hydroxylation of D-camphor, thus enabling the bacterium to grow on D-camphor as the only carbon source. Its flavoprotein, putidaredoxin reductase, is a FAD containing, strictly NADH-dependent ferredoxin reductase. The iron sulfur protein, putidaredoxin, is a [2Fe–2S] ferredoxin which shuttles two electrons one at a time from putidaredoxin reductase to P450cam [19].

An example of the mitochondrial P450 enzyme of class I is CYP11A1 which is also known as P450scc (side-chain cleavage). This enzyme is involved in the conversion of cholesterol into pregnenolone, which constitutes the initial step of steroid hormone biosynthesis.

Members of the Class II P450 enzymes receive their electrons from NADPH via the membrane bound cytochrome P450 reductase (CPR). The latter reductase contains FAD and flavin mononucleotide (FMN) binding domains that shuttle the electrons from NADPH to the P450 haem which is also membrane bound (Figure 1B). The reductase has evolved as a fusion of two ancestral proteins and shows in the N-terminal region homology with the FMN-containing bacterial flavodoxins while the C-terminal portion is homologous with the FAD-containing ferredoxin NADP+ reductases and with NADH-cytochrome b5 reductase [20]. This class includes microsomal P450s examples of which are the human hepatic cytochromes P450 with their large number and wide variety of substrates including therapeutic drugs and xenobiotics.

Class VII enzymes have a FMN containing reductase which is fused in a single polypeptide chain with a ferredoxin-like centre and a P450 domain (Figure 1D). The electrons are relayed to the P450 through a FMN centre and a [2Fe2S] ferredoxin-like component. The best-known example of this class is the cytochrome CYP116B2 (P450RhF) from *Rhodococcus sp*. strain NCIMB 9784. It comprises a N-terminal P450 domain fused to a FMN- and 2Fe-2S-containing reductase domain. The reductase (RhFRed) displays similarity to the phthalate family of oxygenases and transfers electrons from NADPH to the haem domain [18, 21].

Finally, Class VIII P450s are enzymes containing binding sites for haem, FAD and FMN in a single-polypeptide chain (Figure 1C). These enzymes require NADPH as a source of electrons and represent a naturally fused system made up of the components of the class II system but with the added advantage of being self-sufficient. Members of this group include the bacterial P450s. The most intensively studied member of this class is CYP102A1 (P450BM3) of *Bacillus megaterium* [22]. It is composed of a haem domain (BMP) connected via a short protein linker to a diflavin reductase domain (BMR) containing the cofactors FAD and FMN. This enzyme catalyses the NADPH-dependent hydroxylation of medium and long-chain saturated fatty acids displaying the highest monooxygenase activity of any P450 system investigated to date. Due to its structure, P450BM3 has been and still is used as a model of the human hepatic P450 enzymes.

**3. Chimeric P450s to improve or expand their catalytic properties**

The large substrate diversity and catalytic versatility of cytochrome P450 enzymes has led to an immense interest in the engineering of these enzymes for various biotechnological applications. The literature reports multiplecases in which functional cytochrome P450 fusion proteins between natural and artificial redox partners have been reconstituted in order to allow the expression and purification of high levels of catalytically self-sufficient protein. These catalytically self-sufficient P450s have a wide range of exciting possible applications. Bacteria containing heterologously expressed cytochrome P450 fusion proteins will aid in the understanding of human drug metabolism, the consequences of drug-drug interactions and the activation of carcinogens. There is also the possibility of exploiting these enzymes as biocatalysis [23, 24] or using hosts containing these proteins for bioremediation purposes or biosensing [9, 10, 25-27]. Furthermore, the careful selection of fusion partners could allow the creation of cytochrome P450 fusion proteins that have improved properties in comparison to the parent enzyme such as solubility, higher activity and enhanced coupling of co-factor consumption.

**3.1 Microsomal P450 fusion constructs with microsomal reductases**

The earliest reported microsomal P450/CPR fusion protein was the rat CYP1A1/rat NAPDH cytochrome P450 reductase fusion protein constructed by Murakami and co-workers [28]. Rat cytochrome P450 reductase (CPR) is a membrane bound enzyme and construction of a fusion protein required the removal of the N-terminal membrane anchor of the CPR. The truncated form of rat CPR was fused to rat CYP1A1, the enzyme was expressed in the holo form and it was shown to be active against 7-ethoxycoumarin. Treatment of the microsomes with trypsin liberated the CPR but not the CYP1A1 indicating that there was a proteolytically sensitive loop between the two proteins. The purified fusion protein retained activity, which did not increase upon addition of CPR suggesting that efficient electron transfer was occurring between the two domains within the fusion. The use of the truncated form of rat CPR was further validated by later studies that demonstrated that CPR could still act as a cytochrome c reductase without its N-terminal membrane anchor and could function outside the membrane environment [29]. In light of this, the same research group constructed further fusions between bovine CYP17A1 (P450c17) or bovine CYP21 (P450c21) and yeast CPR that were active towards their respective substrates [30, 31]. These studies initiated the construction of a number of microsomal P450/reductase fusion proteins that resulted in catalytically self-sufficient enzymes with improved properties.

Estabrook and co-workers constructed fusions between bovine CYP17A and rat CPR and between rat CYP4A1 and rat CPR [32]. The purified fusion proteins were active and demonstrated NADPH oxidation rates that were tightly coupled to substrate hydroxylation. This group applied the same fusion protein principal to human CYP3A4 to create a CYP3A4/rat CPR fusion protein [33]. The purified protein exhibited a requirement for lipid, detergent and cytochrome *b*5 for the hydroxylation of testosterone and N-oxidation of nifedipine but not for the N-demethylation of erythromycin where these additions actually inhibited product formation. In a later study by the same research group on the fusion, it was also found that addition the of cytochrome *b5* stimulated testosterone hydroxylation by reducing hydrogen peroxide formation and therefore uncoupling [34]. Furthermore, addition of purified rat CPR in the presence of lipid, detergent and cytochrome *b5* stimulated testosterone hydroxylation approximately 10 folds indicating that electron transfer within the protein was not efficient. Halpert and colleagues [35] used the same linker as Estabrook’s group [32] in order to construct fusions of dog P450 2B11 and rat liver NADPH-cytochrome P450 reductase in order to facilitate mutagenesis analyses of P450 2B11. Sonicated whole cell lysates of *E. coli* cells expressing the fusion protein were able to catalyse the 16-hydroxylation of androstendione in the absence of added reductase, and had activities and metabolite profiles similar to those of purified and reconstituted P450 2B11 enzyme preparations. They went on to use the fused enzyme to study the importance of a specific charged amino acid for enzymatic activity of P450 2B11.

A similar approach was used to engineer a human CYP1A1/rat CPR fusion protein that was active against 7-ethoxyresofurin, Benzo[a]pyrene and zoxazolamine by Guengerich’s group [36]. Activity of the fusion was not increased in the presence of added CPR, cytochrome *b*5 or phospholipids. Additionally the fusion could not transfer electrons to added CYP1A1 suggesting that electron transfer was intra-domain. The group later extended the approach to human CYP1A2 and created a human CYP1A2/rat CPR fusion protein that, when purified, catalysed 7-ethoxyresofurin O-deethylation and phenacetin O-deethylation in the presence of NADPH and phospholipids [37].

Kondo and co-workers engineered a fusion between rat CYP1A1 and yeast CPR that catalysed 7-ethoxyresofurin O-deethylation [38]. In the study both mixed and fused systems had comparable activities that were found to decrease with increasing ionic strength suggesting an ionic interaction between the P450 and reductase domain that was being reduced upon increasing ionic strength of the buffer. A similar construct between human CYP3A4 and yeast CPR was found to be more active at 6β-hydroxylation of testosterone than a mixed system [39, 40]. However, coupling efficiency between NADPH utilization and substrate hydroxylation in the absence of cytochrome *b*5 was only 10 % and increased to 50 % in the presence of cytochrome *b*5 for both fused and mixed systems.

In another example, a fusion between human CYP2D6 and its redox partner human CPR was created by Deeni and co-workers [41] where the enzyme was able to catalyse bufuralol hydroxylation and dextromethorphan O-demethylation. However, activity was enhanced 3-fold by the addition of phospholipids and purified NADPH-P450 reductase suggesting that electron transfer was intermolecular.

Reports of other P450 fusions including CYP11A1-adrenodoxin reductase, CYP11A1- adrenodoxin-adrenodoxin reductase and CYP11A1-adrenodoxin reductase-adrenodoxin, with the latter producing considerably more pregnenolone than a control triple transfection in COS-1 cells, have been described [42]. In the same way, different human and bovine CYP11B1 fusions with adrenodoxin and adrenodoxin reductase (bovine) were created and successfully expressed in COS-1 cells, although even the best fusion protein had less activity than CYP11B1 alone, receiving electrons from co-expressed reductase proteins [43].

**3.2 Microsomal P450 fusion constructs with bacterial reductases**

The construction of fusion proteins has not been limited to class II P450 systems and chimeras have also been made using components of class I and class VIII P450s. Microsomal cytochromes P450 have also been fused to domains from the bacterial enzymes CYP102A1 (P450BM3) and CYP101 (P450cam). A fusion protein comprising the rat CYP2C11 fused to BMR (the reductase domain of CYP102A1) *via* a Pro-Ser-Arg linker was found to catalyse the metabolism of arachidonic acid [44]. However, its activity was lower than both a reconstituted system containing CYP2C11 and rat CPR and a fused system between CYP2C11 and rat CPR. Furthermore, activity of the CP2C11/BMR fusion was greatly enhanced upon addition of purified reductase either of mammalian or bacterial origin indicating poor intra-molecular electron transfer.

Using the molecular Lego approach [9, 10], our laboratory has created fusion proteins consisting of the human CYP2E1, CYP2C9, CYP2C19 and CYP3A4, and monkey 2C20 fused to the reductase domain of CYP102A1 (BMR) [11, 12, 45], as shown in Figure 2. The CYP2E1/BMR, CYP2C9/BMR, CYP2C19/BMR, CYP3A4/BMR and CYP2C20/BMR chimeras were all active and correctly folded in the absence of detergent and in comparison with the parent P450 enzyme, these chimeras showed greatly improved solubility properties. The chimeras were catalytically self-sufficient and presented turnover rates similar to those reported for the native enzymes in reconstituted systems, unlike previously reported mammalian cytochrome P450 fusion proteins. Furthermore, the specific activities of these chimeras were not dependent on the enzyme concentration present in the reaction buffer and did not require the addition of accessory proteins, detergents or phospholipids to be fully active. However, in *in vitro* studies the coupling efficiency, within this chimera, between NADPH utilization and substrate demethylation was not increased [46].

Our group also used the same approach [9, 10] for fusing the human CYP3A4 with flavodoxin (from *Desulfovibrio vulgaris*) as the electron transfer domain [47] (Figure 2). In this case it was shown how regulating the electron flow between the human CYP3A4 and electrode surfaces via the flavodoxin enhanced the coupling efficiency and catalytic activity of the immobilised CYP3A4 towards its substrate erythromycin [47]. In a further advancement of the same electrochemical principle, different polymorphic variants of 2C9, namely 2C9.1, 2C9.2 and 2C9.3 were fused with flavodoxin in an amperomteric platform. The latter platform allowed for the determination of small but significant differences in metabolism of S-warfarin [48]. Such determinations of drug clearance properties of polymorphic P450 enzymes have direct relevance in healthcare specially in the context of “Personalised medicine”.

**3.3 Plant P450 fusions with bacterial reductases**

The focus of plant P450 fusion protein research has been to engineer herbicide tolerance in plants and to also harness their great biosynthetic activities for human purposes. Lamb and co-workers constructed a fusion protein between CYP71B1 from *Thlaspi arvensae* and the CPR domain of *Catharanthus roseus* to study the effect of glyphosate (Roundup) on CYP71B1 [49] The study showed that glyphosate inhibited the turnover of the polycyclic aromatic hydrocarbon benzo[a]pyrene (another herbicide) by the enzyme.

The expression of plant P450 fusion proteins in bacteria allows for the production of medically important compounds that plants produce naturally at low levels. Schroder and co-workers constructed a fusion protein between *Catharanthus roseus* CYP71D12 and its reductase [50]. The fusion enzyme was successfully expressed in bacteria and the protein exhibited tabersonine 16-hydroxylase activity. Tabersonine 16-hydroxylase is the first enzyme in a pathway that produces the medically important bisindoles vinblastine and vincristine, both of which have uses in the treatment of leukaemia. These compounds are produced naturally in plants at very low levels (0.0005% of plant mass) and chemical synthesis due to their complex structure is costly and difficult. Harnessing the biosynthetic capability of P450s to create an artificial synthetic pathway allows the mass production of these compounds.

The concept of engineering herbicide resistance into plants using P450s has not been limited to plant P450 fusions. Ohkawa’s group engineered a rat CYP1A1/yeast CPR fusion protein in tobacco plants that was shown to provide the plants with resistance to the herbicide chlortoluron [51]. The same research group also constructed a fusion protein consisting of rat CYP1A1 fused to the iron sulphur maize ferredoxin (Fd) protein and the FAD containing pea ferredoxin NADP+ reductase (FNR) [52]. The most active fusion, CYP1A1-FNR-Fd, when expressed in yeast was active towards 7- ethoxycoumarin and the herbicide chlortoluron.

**3.4 Bacterial P450 fusions with bacterial reductases**

Another chimeric cytochrome P450 constructed using the molecular “Lego” approach [9, 10] connected the non-physiological redox proteins, the haem domain of CYP102A1 (from *Bacillus megaterium*) and the flavodoxin from *Desulfovibrio vulgaris*, creating a BMP-FLD fusion protein which showed improved electrochemical and catalytic properties [53].

Montellano’s group [54] produced a number of fusion proteins between CYP101 (P450cam) and its two reductase components putidaredoxin (an iron sulphur protein; Pd) and putidaredoxin reductase (a FAD protein; PdR). Of the various constructs the PdR-Pd- P450cam construct was found to have the highest activity with the substrate camphore.

Misawa and colleagues [55] demonstrated that CYP101A from *Pseudomonas putida*, CYP203A from an environmental metagenome library and CYP153A from *Alcanivorax borkumensis* SK2 could be expressed in *E. coli* as soluble and functionally active fusion proteins with RhFRED (the reductase domain of P450RhF from *Rhodobacter* sp. NCIMB 9784). For CYP153A, this allowed the direct identification of the function of this specific P450 as alkane 1-monooxygenase [55]. Sherman and co-workers [56] using the same reductase characterised the chimeric single component bacterial biosynthetic P450 PikC fused to RhFRED. PikC catalyzes the final hydroxylation step toward the 14-membered ring macrolactone narbomycin to produce methymycin/neomethymycin and pikromycin as major products. However, since the native redox partner of PikC remains unknown, its *in* v*itro* activity had previously depended on expensive spinach ferredoxin reductase (Fdr) and ferredoxin (Fdx). The self-sufficient PikC-RhFRED chimeric protein showed significantly improved catalytic activity compared to wild type PikC in the presence of the exogenous redox partners [56].

In order to improve the efficiency of the chimeric system used by Misawa and co-workers [55], Robin and colleagues [57] produced seven chimeric enzymes of P450cam-RhFRED with linkers of different lengths. One of the latter constructs resulted in a 10-fold improvement for the whole-cell conversion of camphor to 5-exo-hydroxycamphor.

Another study reported the functional expression of CYP153A genes from various environments by incorporating them into a framework consisting of the N and C termini of homologCYP153A13a and fusion to the reductase domain of CYP116B2 [58]. The researchers also showed the fusion protein-dependent *in vivo* conversion of n-alkanes, cyclohexane, 1-octene, n-butylbenzene and 4-phenyl-1-butene [58].

Kim and colleagues [59] have recently reported a self-sufficient chimera of bacterial CYP51 with an Fe-S containing NAD(P)H reductase. The two proteins were fused using two different linkers, one from P450 BM3 (*B. megaterium*) sequence and the other from P450foxy (*Fusarium oxysporum*). Both resulting chimeric proteins catalysed demethylation of lanosterol more efficiently, 35-fold higher, compared to CYP51 and Fe-S containing NAD(P)H reductase alone.

The research group of Arnold have reported the construction of a synthetic family of more than 3000 properly folded cytochrome P450 haem domains [60] assembled by structure-guided recombination of the haem domains of CYP102A1 from *Bacillus megaterium* (A1) and its homologs CYP102A2 (A2) and CYP102A3 (A3). Reconstitution of the chimeric CYP102A haem domains with the three parental reductases generated functional monooxygenases in all cases, fusion to a reductase was never detrimental to activity, and swapping the reductase never completely inactivated the enzyme. Surprisingly, the reductase was able to influence substrate specificity; for example A1 was three-fold more active towards 2-phenoxyethanol if BMR was replaced with the A2 reductase domain, but showed a three-fold decrease in activity towards 12-p-nitrophenoxydodecanoic acid [61].Furthermore, they went on to show that members of their synthetic P450 family exhibited diverse activities and specificities, including activities toward substrates that were not accepted by the parent P450s and therefore these enzymes could be useful for synthesis of drug metabolites, which are needed for toxicity testing and drug discovery [61].

**4. Conclusions and outlook**

The discovery of novel P450 systems (http://drnelson.uthsc.edu/cytochromeP450.html) has resulted in the identification of a wide variety of electron transport chains. Although initially the majority of the research on P450 systems has been focused on the substrate specificity and the resulting activity of the P450s, today more attention is dedicated to the variety of redox partners and the possibility of utilizing them for enhancing the P450 catalytic properties. The availability of different redox partners together with protein engineering approaches for making self-sufficient fusion systems, promises to generate new artificial enzymes for biotechnological exploitation in the fields of design and synthesis of metabolites, pro-drug activation, toxicology research, bioremediation, plant herbicide tolerance, bioelectrocatalysis and biosensing.

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Table 1

Classification of P450 systems by topology of the redox chain [adapted from ref 18].

|  |  |
| --- | --- |
| P450 Class | Electron transport chain |
| Class I | NAD(P)H ▸ [FdR] ▸[Fdx] ▸ [P450] |
| Class II | NAD(P)H ▸[CPR] ▸[P450] |
| Class III | NAD(P)H ▸[FdR] ▸[Fld] ▸[P450] |
| Class IV | Pyruvat, CoA ▸[OFOR] ▸[Fdx] ▸[P450] |
| Class V | NADH ▸[FdR] ▸ [Fdx–P450] |
| Class VI | NAD(P)H ▸[FdR] ▸[Fld–P450] |
| Class VII | NADH ▸[PFOR–P450] |
| Class VIII | NADPH ▸[CPR–P450] |
| Class IX | NADH ▸[P450] |
| Class X | [P450] |

Abbreviated protein components contain the following redox centres:

Fdx (iron–sulphur-cluster);

FdR, Ferredoxin reductase (FAD);

CPR, cytochrome P450 reductase (FAD, FMN);

Fld, Flavodoxin (FMN);

OFOR, 2-oxoacid:ferredoxin oxidoreductase (thiamine pyrophosphate, [4Fe–4S] cluster); PFOR, phthatate-family oxygenase reductase (FMN, [2Fe–2S] cluster).

Table 2

Different P450 fusion or chimeric proteins constructed to date.

|  |  |  |  |
| --- | --- | --- | --- |
| P450 | Reductase | Substrate | Reference |
| Microsomal fusions | | | |
| Rat CYP1A1 | Rat CPR | Ethoxycoumarin | [28] |
| Bovine CYPc17A | Yeast CPR | Progesterone | [30] |
| Bovine CYPc21 | Yeast CPR | Progesterone,  17 alpha-hydroxyprogesterone | [31] |
| Bovine CYP17A1 | Rat CPR | Progesterone | [32] |
| Rat CYP4A1 | Rat CPR | Lauric acid | [32] |
| Human CYP3A4 | Rat CPR | Testosterone | [33] |
| Dog CYP2B11 | Rat CPR | Androstendione | [35] |
| Human CYP1A1 | Rat CPR | 7-ethoxyresofurin Benzo[a]pyrene | [36] |
| Human CYP1A2 | Rat CPR | 7-ethoxyresofurin | [36] |
| Rat CYP1A1 | Yeast CPR | 7-ethoxycoumarin | [38] |
| Human CYP3A4 | Yeast CPR | Testosterone | [39, 40] |
| Human CYP2D6 | Human CPR | Bufuralol, dextromethorphan | [41] |
| Human CYP11A1 | AdR + Ad | Pregnenolone | [42] |
| Human CYP11B1  Bovine CYP11B1 | AdR+Ad (bovine) | Deoxycorticosterone | [43] |
| Rat CYP2C11 | BMR | Arachidonic acid | [44] |
| Human CYP2E1 | BMR | Chlorzoxazone, p-nitrophenol | [11] |
| Human CYP2C9 | BMR | Diclofenac | [12] |
| Human CYP2C19 | BMR | Omeprazole | [12] |
| Human CYP3A4 | BMR | Erythromycin | [12] |
| Monkey CYP2C20 | BMR | Paclitaxol, Amodiaquine | [45] |
| Human CYP3A4 | Fld (*D. vulgaris*) | Erythromycin | [47] |
| Human CYP2C9 | Fld (*D. vulgaris*) | (S)-warfarin | [48] |
| Plant fusions | | | |
| CYP71B1  *T. arvensae* | CPR (*C. roseus*) | Benzo[a]pyrene | [49] |
| CYP71D12  *C. roseus* | CYP71D12  reductase | Tabersonine | [51] |
| Rat CYP1A1 | Maize Fd + pea FNR | 7-ethoxycoumarin, chlorotoluron | [52] |
| Bacterial fusions | | | |
| CYP102A1 | Fld (*D. vulgaris*) | p-nitrophenol | [53] |
| CYP101 | Pd and PdR | Camphor | [54] |
| CYP101A  CYP203A  CYP153A | RhFRED | Camphor  4-hydroxybenzoate  Octane | [55] |
| CYPPikC | RhFRED | Narbomycin | [56] |
| CYP101 | RhFRED | Camphor | [57] |
| CYP153A13a | CYP116B2 reductase | Cyclohexane  1-octene  n-butylbenzene  4-phenyl-1-butene | [58] |
| CYP51 | Fdx reductase | lanosterol | [59] |
| CYP102A1  CYP102A2  CYP102A3 | Parental reductases in different combinations | 2-phenoxyethanol  12-p-nitrophenoxydodecanoic acid | [61] |

Abbreviated protein components contain the following redox centres:

Ad, adrenodoxin (iron–sulphur-cluster);

AdR, adrenodoxin reductase (FAD);

BMR, reductase domain of CYP102A2 (FAD, FMN);

CPR, microsomal cytochrome P450 reductase (FAD, FMN);

Fdx Ferredoxin (iron–sulphur-cluster);

FdR, Ferredoxin reductase (FAD);

Fld, Flavodoxin (FMN);

Pd, putidaredoxin (iron–sulphur-cluster);

PdR, putidaredoxin reductase (FAD);

RhFRED, phthatate-family oxygenase reductase (FMN, [2Fe–2S] cluster).

**Figure Legends**

**Fig. 1**: Representative topologies of cytochrome P450 enzymes with the common haem domain/protein but different reductase domains/proteins. (A) Class I- three separate proteins: FAD-containing reductase, ferredoxin ([2Fe-2S] cluster) and haem; (B) Class II- two membrane-bound proteins: di-flavin P450 reductase (FAD and FMN) and haem; (C) Class VIII- natural fusion of di-flavin reductase (FAD and FMN) and haem via an amino acid linker; and (D) Class VII- natural fusion of a phthalate dioxygenase reductase (FMN and [2Fe-2S] cluster) and haem via an amino acid linker.

**Fig. 2**: The Molecular Lego approach [10], fusion of human P450 haem with bacterial reductases: soluble and self-sufficient chimera with reductase domain of cytochrome P450 BM3 (top); soluble chimera with flavodoxin (bottom).



