Bioresources and Bioprocessing

REVIEW

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Bacterial cytochrome P450-catalyzed regio- and stereoselective steroid hydroxylation enabled by directed evolution and rational design

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Abstract

Steroids are the most widely marketed products by the pharmaceutical industry after antibiotics. Steroid hydroxylation is one of the most important functionalizations because their derivatives enable a higher biological activity compared to their less polar non-hydroxylated analogs. Bacterial cytochrome P450s constitute promising biocatalysts for steroid hydroxylation due to their high expression level in common workhorses like *Escherichia coli*. However, they often suffer from wrong or insufficient regio- and/or stereoselectivity, low activity, narrow substrate range as well as insufficient thermostability, which hampers their industrial application. Fortunately, these problems can be generally solved by protein engineering based on directed evolution and rational design. In this work, an overview of recent developments on the engineering of bacterial cytochrome P450s for steroid hydroxylation is presented.

Keywords: Biocatalysis, Cytochrome P450, Steroid hydroxylation, Regioselectivity, Stereoselectivity, Directed evolution, Rational design

Introduction

Cytochrome P450 (CYPs) belong to a superfamily of heme-containing enzymes which typically act as monooxygenase, catalyzing the reductive scission of molecular oxygen, thereby introducing one oxygen atom into the substrate, whereas the second oxygen atom is reduced to water (Urlacher and Girhard 2012). To achieve high catalytic activity, CYPs must be associated with redox partner proteins which can transfer electrons from NAD(P)H via flavins (FMN, FAD) or Fe–S clusters to the P450 heme center. Based on the different electron transport types, P450s can be classified into three-, two- or one-protein systems (Fig. 1). CYPs are versatile biocatalysts and are capable of catalyzing regio- and stereoselective functionalization of non-activated hydrocarbons under mild reaction conditions (Guengerich et al. 2016), thus accomplishing chemical transformations that are of significant challenge in synthetic organic chemistry (Renault et al. 2014; Li et al. 2002). Since their discovery, CYPs have attracted researchers from different areas of science including metabolic engineering (Renault et al. 2014), synthetic biology (Girvan and Munro 2016), natural product discovery (Podust and Sherman 2012), drug metabolism (Romdhane et al. 2012; Guengerich 2001, 2008), toxicology (Behrendorff and Gillam 2016), bioremediation (Kellner et al. 1997) and plant protection (Morant et al. 2003). CYPs have also led to the generation of commercial products (Guengerich 2002).

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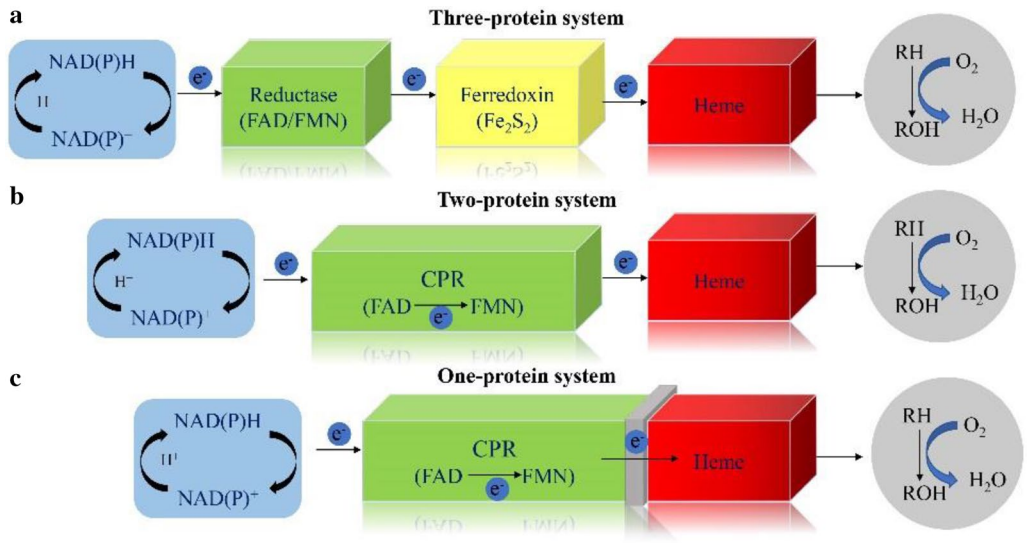

Fig. 1 Different redox systems for electron transfer in CYPs. **a** Three-protein systems: P450 and reductase can either be soluble or membrane-bound. **b** Two-protein systems: cytochrome P450 reductase (CPR) and P450 are membrane-bound. **c** One-protein system: soluble or membrane-bound systems formed by fusions of a CPR-like reductase and P450

As times goes on, more and more CYPs have been discovered, identified, characterized, and investigated for their ability to catalyze many types of oxidations of a vast number of substrates (Mallinson et al. 2018; O'Reilly et al. 2013; Ren et al. 2015), including, inter alia, hydroxylation (Lewis et al. 2011), epoxidation (Li et al. 2013, 2015; Guengerich 2003), sulfoxidation, dealkylation (Hartwig 2015) and C–C bond cleavage, etc. (Bernhardt and Urlacher 2014; Munro et al. 2007; Li et al. 2016a; Coelho et al. 2013; Wang et al. 2014; Prier et al. 2017; Mallinson et al. 2018).

Steroid-based drugs are the most marketed drugs after antibiotics, of which CYP-mediated steroid hydroxylation is very important for pharmaceutical applications because this particular modification enhances their biological activity (Li et al. 2002; Fernandes et al. 2003; Donova and Egorova 2012). Current production methods of hydroxylated steroids mainly rely on fungal strains, however, bioconversion with wild-type (WT) CYPs can result in a mixture of products. For example, conversion of 11-deoxycortisol to hydrocortisone by *Curvularia lunata* is accompanied with an unspecific 14α hydroxylation (Donova 2017). On the other hand, mammalian P450s exhibit high regio- and stereoselectivity, but their activity is extremely low. For these reasons, alternative sources of CYPs are needed.

So far, a number of CYPs involved in steroid biosynthesis from different origins (plants, animals and microorganisms) have been identified using bioinformatics (Nelson 2018). Compared to eukaryotic ones, bacterial P450s possess many advantages. They can be overexpressed in high amounts in soluble form and thus are convenient for genetic diversification and purification (Bernhardt and Urlacher 2014). Moreover, bacterial CYPs are generally much more active than eukaryotic ones; typically, turnover numbers go from ten to a few hundred molecules per min. Due to the above reasons, CYPs from different bacterial species capable of hydroxylating steroids with different regio- and stereoselectivity have been documented (Fig. 2). A multiple sequence alignment of corresponding steroid-hydroxylating CYPs (Gotoh 1992) showed that the substrate recognition sites (SRS) and some conserved motifs (heme binding, substrate binding, redox partner binding, proton delivery and active center) could display general features of bacterial P450s (Fig. 3). However, such bacterial WT enzymes often suffer from wrong regioselectivity, low activity, narrow substrate range and insufficient thermostability, thus restricting their industrial application. Fortunately, these problems can be generally solved with directed evolution, perhaps the most versatile tool in protein engineering, which consists of iterative rounds of gene mutagenesis, expression and screening or selection. There are two main approaches classified as random (or whole gene) and focused (or structure-based), with error-prone PCR (epPCR), DNA shuffling or recombination and saturation mutagenesis (SM) being the 3 most common methods (Qu et al. 2019). Especially structure-guided

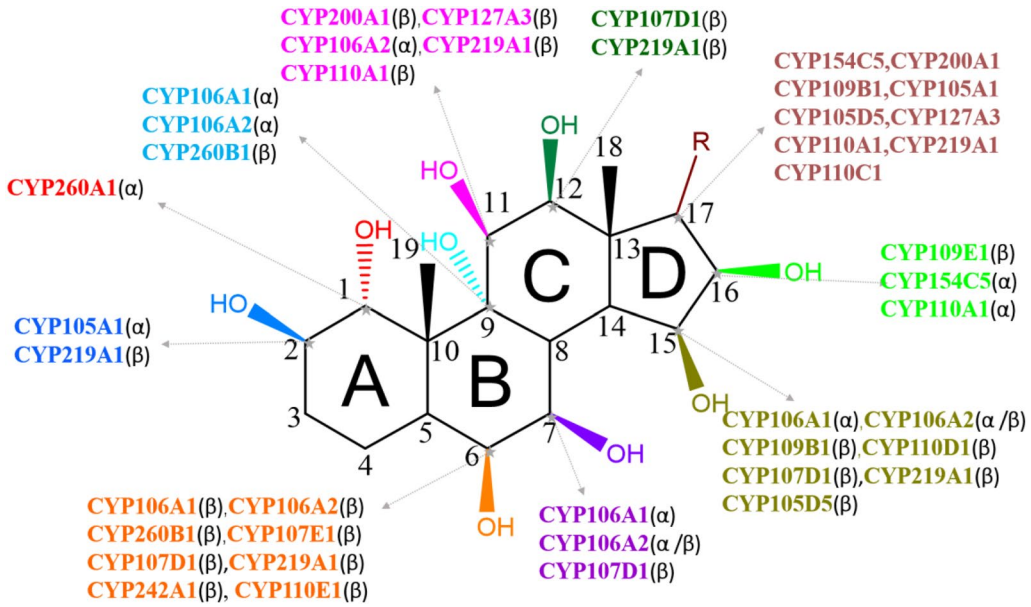

Fig. 2 Steroid hydroxylation by bacterial cytochrome P450 monooxygenases from different species. Typical cases are: CYP106A1 from *Bacillus megaterium* DSM319; CYP106A2 from *B. megaterium* ATCC13368 (Kiss et al. 2015a, b); CYP109B1 from *B. subtilis* 168 (Girhard et al. 2010); CYP109E1 from *B. megaterium* DSM19 (Józwik et al. 2016); CYP154C5 from *Nocardia farcinica* IFM 10152 (Bracco et al. 2013); CYP260A1 and CYP260B1 from *Sorangium cellulosum* Soce56 (Litzenburger and Bernhardt 2017); CYP154C3 from *Streptomyces griseus* SGR1085 (Makino et al. 2014); CYP154C8 from Streptomyces sp. W2233-SM (Dangi et al. 2018) and CYP219A from *Novosphingobium aromaticivorans* DSM12444 (*saro0307*), CYP105A1 from *Streptomyces griseolus* ATCC 11796, CYP107E1 from *Micromonospora griseorubida*, CYP107D1 and CYP127A3 from *Mesorhizobium loti* MAFF303099 *(mlr5876)* CYP110A1, CYP110C1, CYP110D1 and CYP110E1 from *Nostoc* sp. PCC7120 and CYP200A1 from *Bradyrhizobium japonicum* USDA110 (Agematu et al. 2006)

iterative SM has emerged as the optimal choice for evolving selective enzymes needed in the asymmetric synthesis of chiral intermediates and biologically active compounds (Reetz 2004, 2011; Reetz and Carballeira 2007, Reetz and Krebs 2011). Directed evolution has been successfully applied to improve both activity and selectivity of CYPs, for catalyzing not only natural reactions based on focused (Roiban and Reetz 2014) and random (Behrendorff et al. 2015; Kumar and Halpert 2005) approaches, but also non-native reactions (Urlacher and Marco 2019; Zhang et al. 2019). The discovery of new P450 enzymes, their catalytic mechanism in steroid biosynthesis as well as their critical role in steroid metabolism have been extensively investigated and have been the subject of several reviews (Agematu et al. 2006; Donova 2017; Szaleniec et al. 2018). However, works focused on bacterial CYP-catalyzed steroid hydroxylation enabled by protein engineering, have not been reported. In this review, we feature available case studies using different bacterial CYPs for steroid hydroxylation (Table 1), which is discussed according to their phylogenetic classification. Our aim is to understand the activity and selectivity profiles exhibited by WT and engineered CYPs, while exploring their potential for the development of industrial bioprocesses.

CYP102A1: cytochrome $P450_{BM3}$ from *Bacillus megaterium*

CYP102A1 is a fatty acid hydroxylase that can hydroxylate C12-C20 fatty acids as natural substrates, and it is considered as the most active P450 thus far identified (Whitehouse et al. 2008; Narhi and Fulco 1986). Its turnover number for the native fatty acid substrates (C14-C16 is preferred) is about 3000 per min, while most CYPs show 1–300 per min (Bernhardt and Urlacher 2014). It is a single-polypeptide composed of a fusion of the heme domain and NADPH-dependent FMN/FAD reductase. The reductase domain containing two flavin and one NADPH binding region is 65 kDa, whereas the heme domain is 55 kDa, making a large protein of about 120 kDa (Saab-Rincon et al. 2017). Although BM3 is one of the most active CYPs, the application of the WT enzyme is limited by the fact that turnovers involving non-natural substrates often have low NADPH consumption rates and poor coupling efficiency (Whitehouse et al. 2009). Since WT BM3 does not accept bulky steroidal substrates, active-site mutagenesis needs to be implemented (Li et al. 2016b, 2017; Zhou et al. 2019).

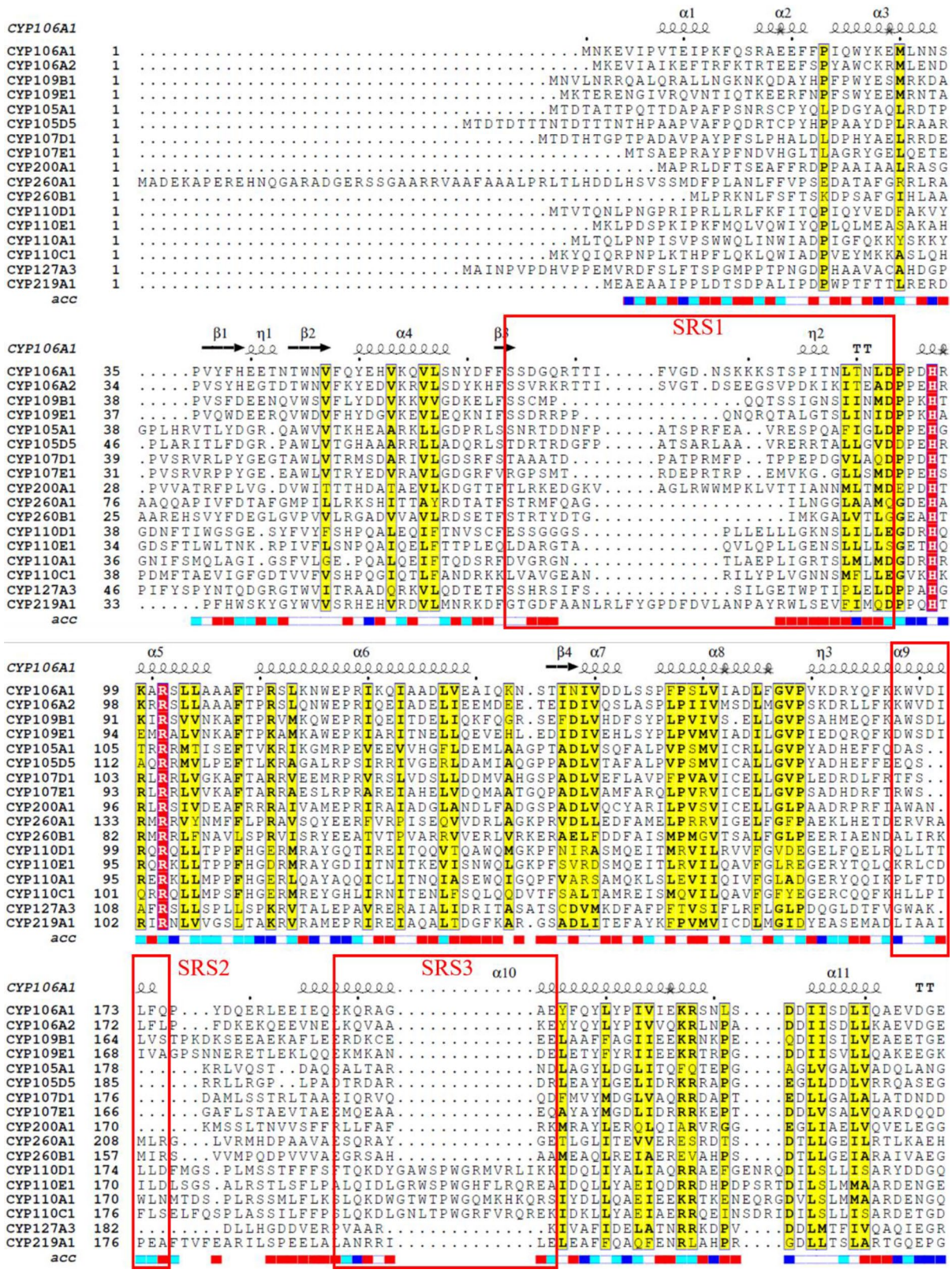

Fig. 3 Multiple sequence alignment of bacterial steroid-hydroxylating CYPs. Substrate recognition sites (SRS) and conservative motifs of bacterial P450s are enclosed in red box and black box, respectively. Conserved and similar residues are highlighted in red and yellow, respectively

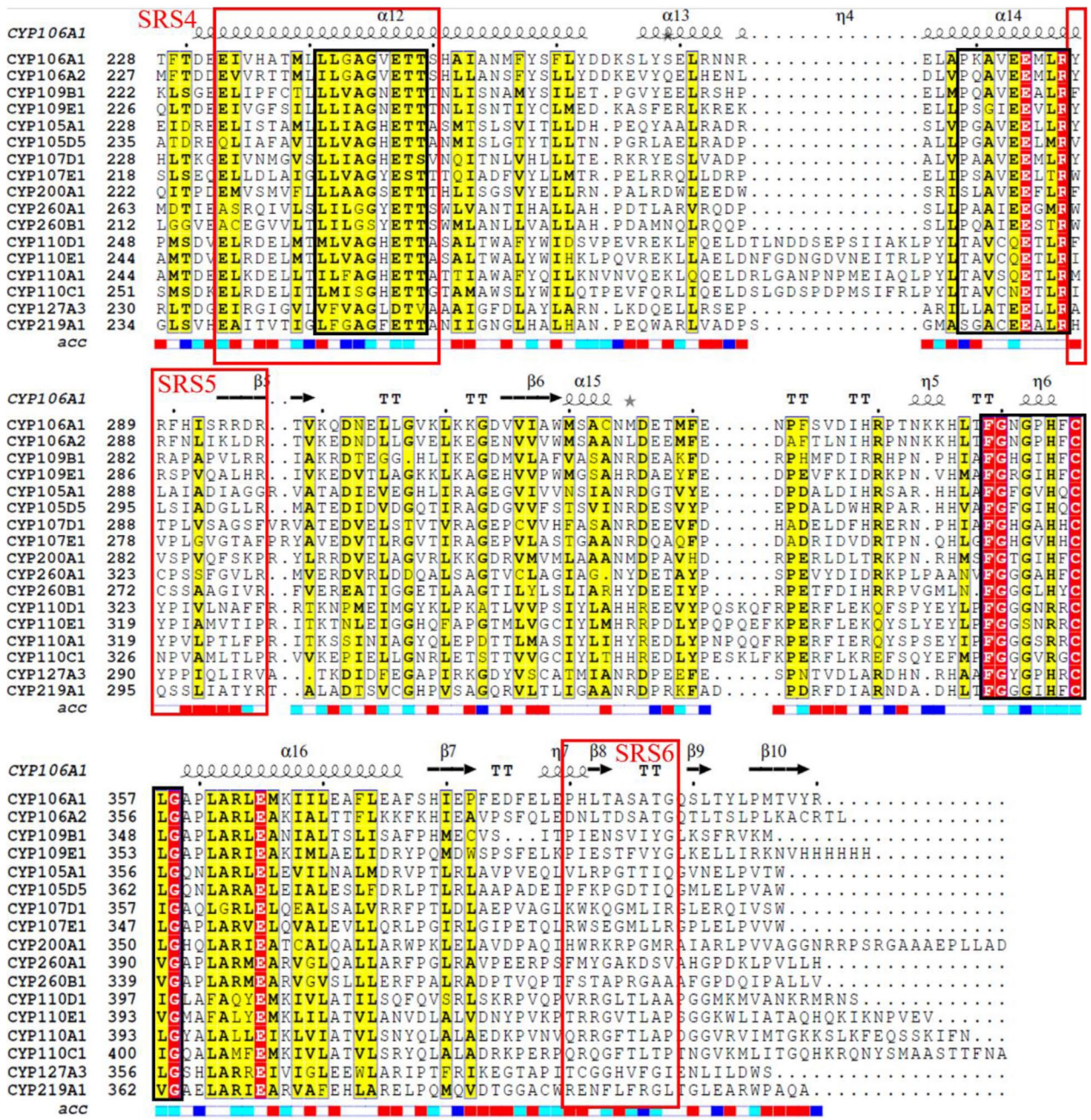

Fig. 3 continued

During the characterization of the substrate specificity of $P450_{BM3}$, it was reported that mutating phenylalanine at site 87 to alanine changes significantly its regiospecificity towards fatty acid hydroxylation (Oliver et al. 1997). Inspired by this finding, variant F87A was first tested for the hydroxylation of testosterone and progesterone. The mutant produces a 52:45 mixture of 2β- and 15β-hydroxytestosterone or 18:82 mixture of 2β- and 16β-hydroxyprogesterone (Kille et al. 2011). Next, the combinatorial active-site saturation test (CAST) was applied iteratively (Qu et al. 2019) on mutant F87A for improving both selectivity and activity. Twenty residues surrounding the binding pocket were divided into nine randomization sites as shown in Fig. 4. Mutagenesis was started on sites A (R47/T49/Y51), B (V78/A82) and C (M185/L188) using NDC,

Table 1 Summary of CYPs variants-catalyzed regio- and stereoselective steroid hydroxylation by directed evolution

CYPs	Mutants	Results	Conversion	Protein engineering strategy
CYP102A1 (*Bacillus megaterium*)	KSA-1	97% 2β-hydroxytestosterone	79	Directed evolution based on CAST, ISM, MLs, MDs, etc. (Kille et al. 2011)
	KSA-1	91% 16β-hydroxyprogesterone	n.d.*	
	KSA-9	100% 2β-hydroxyprogesterone	n.d.*	
	KSA-14	94% 15β-hydroxytestosterone	85	
	M01-A82W	85% 16β-hydroxytestosterone	n.d.	Rational design based on site-directed mutagenesis. (Rea et al. 2012; Venkataraman et al. 2012)
	M01-A82W	88% 16β-hydroxynorethisterone	n.d.*	
	M11-A82W	75% 16β-hydroxytestosterone	n.d.*	
	M11-A82W	78% 16β-hydroxynorethisterone	n.d.*	
	M01-A82W/S72I	81% 16α-hydroxytestosterone	n.d.*	
	WIFI-WC	96% 16β-hydroxyprogesterone	95%	Directed evolution based on CAST, ISM, MLs, MDs, etc. (Acevedo-Rocha et al. 2018)
	WIFI-WC	95% 16α-hydroxyandrostenedione	85%	
	WWV-QRS	92% 16β- hydroxyprogesterone	92%	
	WWV-HMQ	100% 16β-hydroxyandrostenedione	93%	
	WWV-Q	90% 16β-hydroxynandrolone	91%	
	WWV-Q	72% 16β-hydroxyboldenone	71%	
	WWI	91% 16β-hydroxynorethindrone	59%	
	LIFI-CW	98% 16β-hydroxynandrolone	71%	
	LIFI-CW	97% 16β-hydroxyboldenone	70%	
	139-3	100% 1α-hydroxyandrostenedione	37%	Random mutagenesis. (Liu and Kong 2017)
CYP106A2 *(Bacillus megaterium* ATCC 13368)	T89N/A395I	81% 11α- hydroxyprogesterone	24.4- fold than WT	Directed evolution based on CAST, ISM, MLs, MDs, etc. (Nguyen et al. 2012; Nikolaus et al. 2017)
	A243S	88.8% 6β-hydroxyprogesterone	93.1%	
CYP260A1 (*myxobacterium Sorangium cellulosum* Soce56)	S276N	57% 1α- hydroxyprogesterone	n.d.*	Directed evolution based on CAST, ISM, MLs, MDs, etc. (Khatri et al. 2018)
	S276C	48% 1α- hydroxyprogesterone	n.d.*	
	S276I	64% 17α-hydroxyprogesterone	n.d.*	
	S276L	62% 17α-hydroxyprogesterone	n.d.*	
	S276V	58% 17α-hydroxyprogesterone	n.d.*	
CYP260B1 *(Sorangium cellulosum)*	T224A	75% 9α-hydroxy-11-deoxycorticosterone	100%	Rational design based on site-directed mutagenesis (Litzenburger and Bernhardt 2017)

The detailed information of CYP102A1 mutants are as follows: KSA-1 (F87A/A330W), KSA-9 (A82N/F87A), KSA-14 (R47Y/T49F/V78L/A82M/F87A), M01-A82W (R47L/F87V/L188Q/E267V/G415S/A82W), M11-A82W (R47L/F87V/L188Q/E267V/G415S/E64G/F81I/E143G/A82W), M01-A82W/S72I (R47L/F87V/L188Q/E267V/G415S/A82W/S72I), WIFI-WC (R47W/S72I/A82F/S72I/Y51W/L181C), WWV-QRS (R47W/A82W/F87V-L181Q/T436R/M177S), WWV-HQM (R47W/A82W/F87V/Y51H/L181Q), WWV-Q (R47W/A82W/F87V/L181Q), WWI (R47W/A82W/F87I), LIFI-CW (R47L/S72I/A82F/F87I/L188C/A330W) 139-3 (V78A/H138Y/T175I/V178I/A184V/H36Q/E252G/R255S/A290V/A295T/L353V)

NNK and NDC mutagenesis, respectively. Degenerate codon NNK codes for 20 amino acids, while NDC codes for a set of 12 amino acids whose side-chains include polar/nonpolar, charged/uncharged and aromatic/nonaromatic substitutions. After a considerable screening effort of three libraries, mutants showing very high regio- and stereoselectivity were obtained. For example, for testosterone hydroxylation, mutant KSA-1 (F87A/A330W) and KSA-14 (R47Y/T49F/V78L/A82M/F87A) show 97% 2β-selectivity and 94% 15β-selectivity, respectively (Fig. 5a). In the case of progesterone, mutant KSA-9 (A82N/F87A) exhibited 100% 2β-selectivity, whereas mutant KSA-1 (F87A/A330W) displayed 91% 16β-selectivity (Fig. 5b).

In summary, this directed evolution strategy demonstrated how iterative saturation mutagenesis (ISM) enabled the hydroxylation of two steroids with high regio- and stereoselectivity. However, the screening effort was extremely high. About 9000 mutants were screened in order to obtain two selective mutants for each substrate, which represented a screening effort of about 18,000 colonies in a rather low-throughput screening assay. In addition, the activity of mutants towards progesterone is very low. Thus, methodology development was needed to make directed evolution faster and more reliable.

To the best of our knowledge, the first reported engineered P450 for steroid hydroxylation was the triple

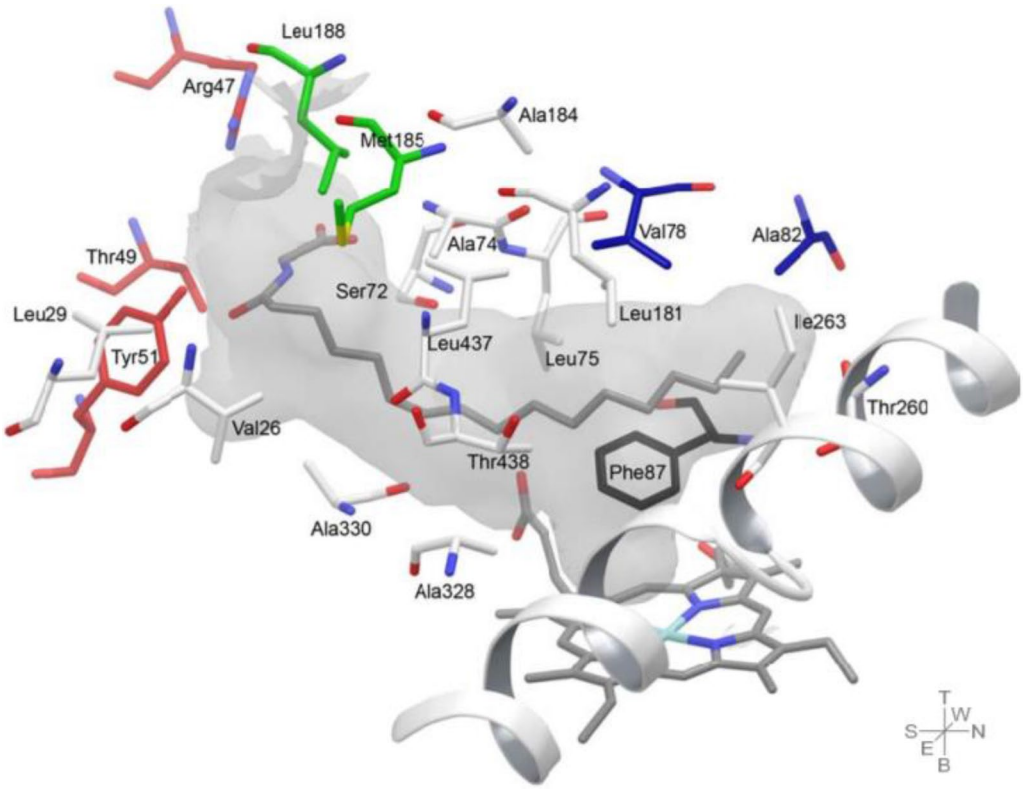

Fig. 4 Active-site residues in the X-ray crystal structure of the N-palmitoylglycine (NPG)-bound form of the heme domain of wild-type $P450_{BM3}$ (1JPZ). The nine sites are labeled as **a** (R47/T49/Y51), **b** (V78/A82), **c** (M185/L188), **d** (S72/A74/L75), **e** (L181/A184), **f** (T260/I263), **g** (A328/A330), **h** (L437, T438) and **i** (V26, L29). The mutant F87A is served as the starting template (Kille et al. 2011)

mutant R47L/L188Q/F87V. It was generated by combining three different mutations via site-directed mutagenesis based on the 3D structure. Earlier, this mutant was engineered by rational design for the oxidation of various substrates (Lussenburg et al. 2005), but it also showed activity on various steroids (testosterone, progesterone, androstenedione and nandrolone), yielding different monohydroxylated metabolites that were not identified (van Vugt-Lussenburg et al. 2006). Later on, the triple mutant was subjected to random mutagenesis, generating variants M01 (R47L/F87V/L188Q/E267V/G415S) and M11 (M01 plus mutations E64G/F81I/E143G) that exhibited increased steroid metabolizing activity. Both variants were tested for their capacity to catalyze the transformation of testosterone and norethisterone, resulting in three and two monohydroxylated products, but with rather low activity and selectivity (de Vlieger et al. 2010; Vottero et al. 2011). It was previously shown that increasing the size and hydrophobicity of active-site residue at position 82 by mutations A82F and A82W in WT $P450_{BM3}$ strongly improves binding affinity for fatty acids and indoles, thus leading to substantially increased catalytic efficiency (Huang et al. 2007). Therefore, Commandeur et al. investigated whether the introduction of the A82W mutation into $P450_{BM3}$ mutants M01 and M11 would affect the activity and/or regioselectivity of steroid hydroxylation (Rea et al. 2012). As expected, mutants M01-A82W and M11-A82W switched selectivity from 15β to 16β for both testosterone and norethisterone. For testosterone, the selectivity for 16β-hydroxytestosterone increased starkly from 22 to 85% and 25 to 75% by mutants M01-A82W and M11-A82W, respectively. In both cases, major products 15β-hydroxytestosterone and 2β-hydroxytestosterone were substantially reduced. In the case of norethisterone, similar results were achieved with 16β-hydroxynorethisterone, selectivity increasing from 42 to 88% and from 58 to 78% with the same mutants, respectively.

Subsequently, Commandeur's group reported that a single active-site mutation at position S72 of variant M01-A82W shifts stereoselectivity of testosterone hydroxylation from 16β to 16α (Venkataraman et al. 2012). Mutation S72V in M01-A82W led to formation of ca. 20% of 16α-hydroxytestosterone, but with 40% 16*β*-hydroxytestosterone still being the major product (Fig. 6). Nevertheless, the authors successfully improved

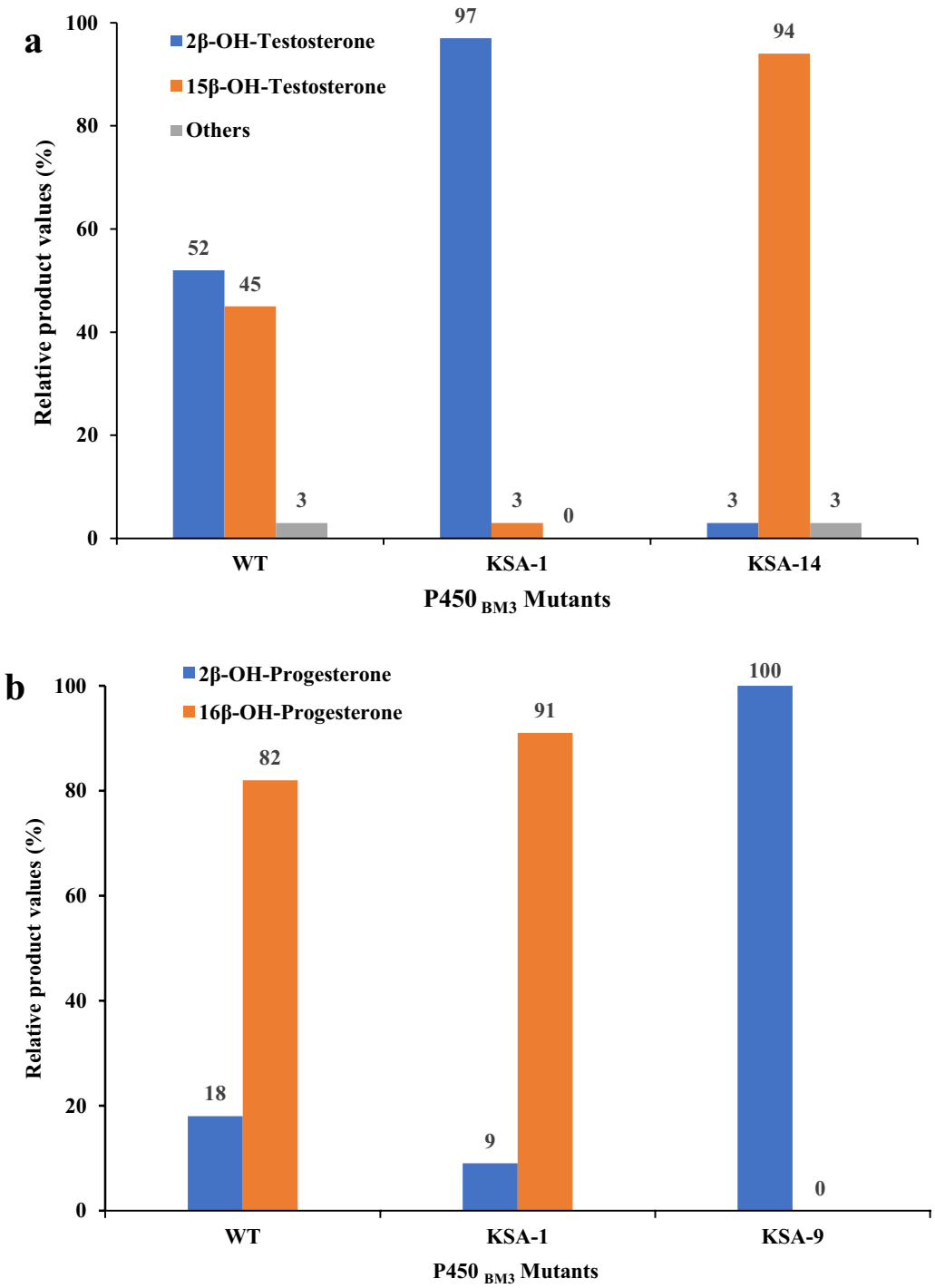

Fig. 5 Relative amounts of hydroxylated products of testosterone (**a**) catalyzed by $P450_{BM3}$ and its mutants KSA-1 (F87A/A330W) and KSA-9 (A82N/F87A); and hydroxylated products of progesterone (**b**) catalyzed by $P450_{BM3}$ and its mutants KSA-1 (F87A/A330W) and KSA-14 (A82N/F87A), respectively. Reaction conditions: 1 mM substrate in KPi buffer pH 7.8, 24 h, 25 °C, resting cells containing expressed P450 mutants

the formation of 16α-hydroxytestosterone with 81% selectivity and only 5% of 16*β*-hydroxytestosterone as minor product by introducing mutation S72I into M01-A82W. However, the activity of the mutant reduced by 2–3 times.

With the three $P450_{BM3}$ mutants (M01-A82W, M11-A82W and M01-A82W/S72I) in hand, *Kong* et al. probed

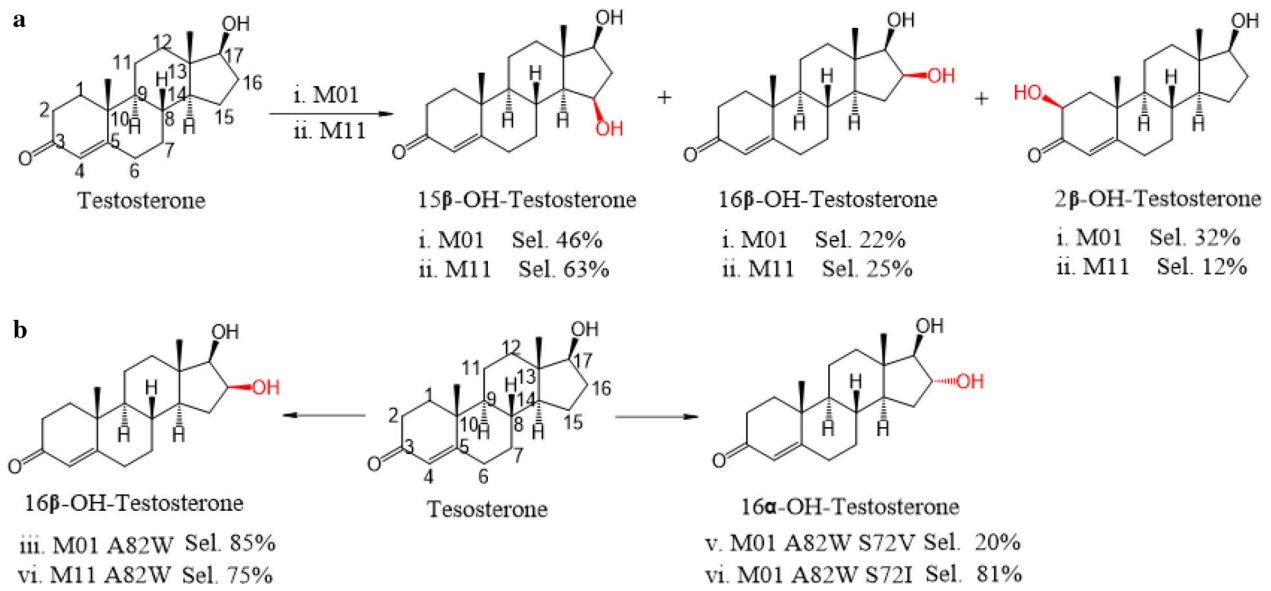

Fig. 6 Mutants of $P450_{BM3}$ from Commandeur's group. The shifts of selectivity of hydroxylated products of testosterone catalyzed by M01, M11 and their mutants M01-A82W, M11-A82W, M01-A82W/S72V. The major hydroxylated products of testosterone by M01 and M11 are shown in **a** and by their mutants are showed in **b**. Reaction conditions: 500 nM purified enzymes containing 0.5 mM testosterone in 250 µL 100 mM KPi buffer pH 7.4, 25 µL NADPH regeneration system containing 2 mM NADPH, 3 mM glucose-6-phosphate and 4 U/mL of glucose-6-phosphate dehydrogenase, 1 h, 24 °C

the steroidal substrate diversity of these variants with various steroid precursors (Liu et al. 2016). Eight steroids including two 3-keto-Δ^4-steroids (testosterone and methyltestosterone) and six 3-hydroxy-5-steroids (cholesterol, β-sitosterol, dehydroepiandrosterone, diosgenin, pregnenolone and ergosterol) were tested. It was found that the three mutants exhibited activity only towards the two 3-keto-Δ^4-steroids, but not on the remaining six 3-hydroxy-Δ^5-steroids.

The aforementioned mutants derived from M01 and M11 display high regio- and stereoselectivity, but low catalytic activity for steroids at position C16. To improve the activity and selectivity simultaneously, it is crucial to solve the issues of diminishing returns and trade-offs in protein engineering (Tokuriki et al. 2012). Moreover, a general problem with the M01-derived mutants is that they are unstable due to disruption of salt bridges and hydrophobic contacts (Geronimo et al. 2016), thus limiting their industrial applications.

Recently, Reetz et al. developed an efficient directed evolution strategy under the guidance of mutability landscape construction to control P450-catalyzed steroid hydroxylation at position 16 with high regio-and diastereoselectivity (Acevedo-Rocha et al. 2018). The authors developed a combined approach concerning the information obtained from mutability landscapes (MLs) and molecular dynamics simulation (MDs) to perform rational iterative saturation mutagenesis (ISM) to escape from diminishing returns and trade-offs with a relatively low screening effort (Fig. 7a). With this approach, two excellent mutants WIFI-WC (R47W/S72I/A82F/S72I-Y51W/L181C) and WWV-QRS (R47W/A82W/F87V-L181Q/T436R/M177S) allowed testosterone hydroxylation at positions 16α and 16β with both activity and selectivity above 92% (Fig. 7b). Compared with M01 and M11-derived variants, the mutants generated in this study greatly improved activity and selectivity. In addition, other steroids (androstenedione, nandrolone, boldenone, and norethindrone) were also activated at position C16 with excellent activity and diastereoselectivity. Overall, the screening effort was about 3000 samples for five different substrates, each with two hydroxylated products. Thus, this combined approach was promising to be an effective method in the directed evolution of other enzymes. More importantly, the functionalization of steroids at position 16 with hydroxyl groups is relevant to develop glucocorticoids for the pharmaceutical industry.

Another study about steroid hydroxylation using $P450_{BM3}$ was reported but using this time variant 139-3 (containing 11 amino substitutions), which was generated in five generations of random mutagenesis with small alkanes as targeted substrates (Glieder et al. 2002). The $P450_{BM3}$ variant 139-3 was used to screen 13 steroid substrates, but it was only able to specifically hydroxylate androstenedione at position 1α with a maximum substrate conversion of 37% (Liu and Kong 2017). Random

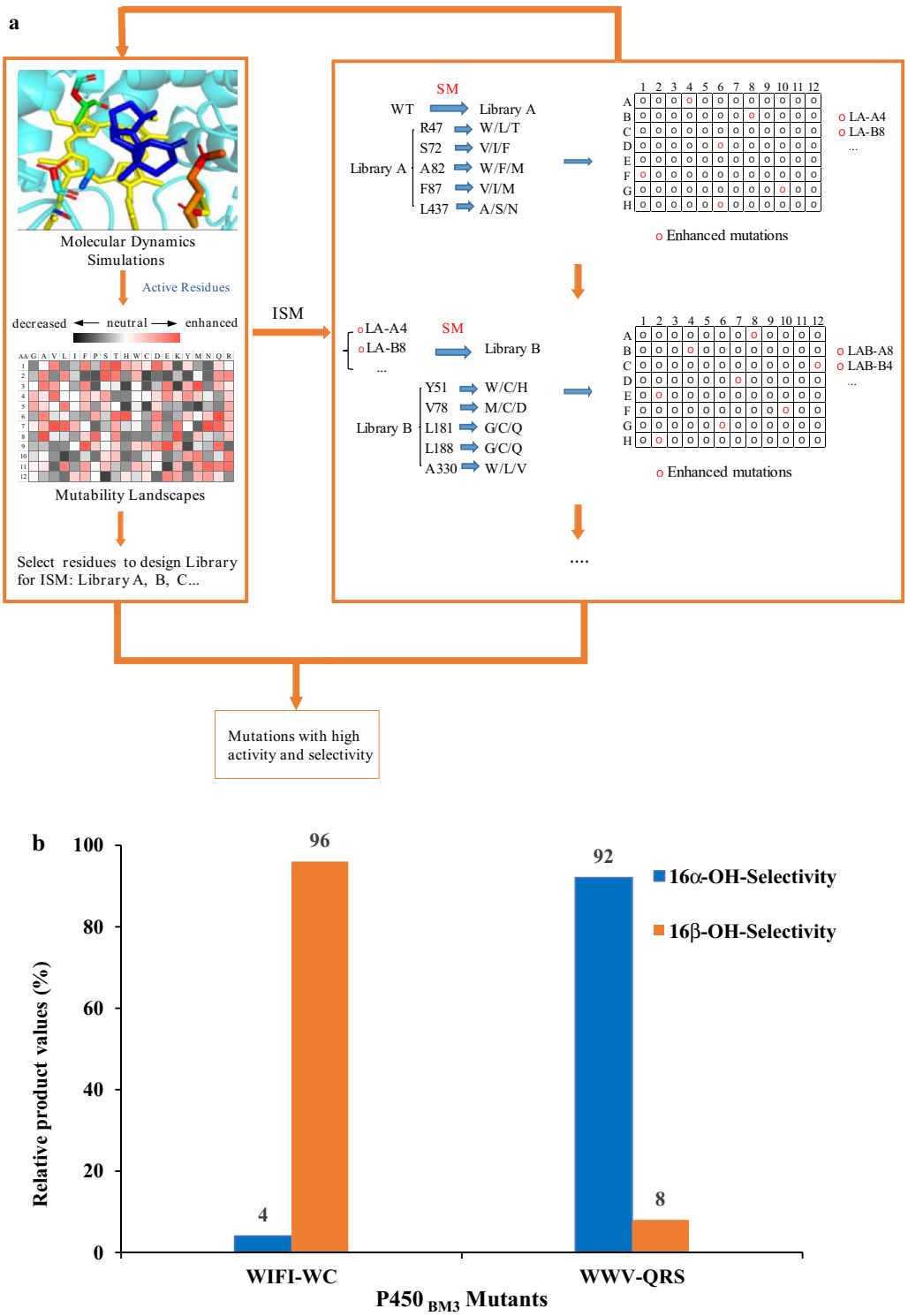

Fig. 7 Controlling regio- and diastereoselectivity at position 16 of various steroids. **a** The directed evolution strategy of $P450_{BM3}$: mutability landscapes (MLs), molecular dynamics simulation (MDS) and iterative saturation mutagenesis (ISM) are combined to design the mutations. **b** Conversion and selectivity of hydroxylated products of testosterone catalyzed by the best two P450 BM3 mutants WIFI-WC (R47W/S72I/A82F/S72I-Y51W/L181C) and WWV-QRS (R47W/A82W/F87V-L181Q/T436R/M177S), respectively. Reaction conditions: 1 mM testosterone in 600 µL, 100 mM KPi buffer pH 8.0 for 24 h, 220 rpm, 37 °C

mutagenesis based on epPCR was performed aiming to further improve activity and substrates specificity of variant 139-3 without a clear target position or type of functionalization, but no improved variants were found. Mutation R379S caused a complete loss of the hydroxylation activity for androstenedione, demonstrating the importance of arginine 379 for enabling 1α-hydroxylated activity of 139-3.

CYP106A2: steroid-specific P450 from *Bacillus megaterium* ATCC 13,368

This bacterial class I enzyme is one of a few tested soluble steroid hydroxylases, which was also identified as a regio- and stereoselective 15β-hydroxylase of 3-oxo-Δ^4-steroids (Berg et al. 1976, 1979; Schmitz et al. 2018). Furthermore, CYP106A2 is a potent hydroxylase of 3-hydroxy-Δ^5-steroids, di- and triterpenoid, although its natural substrate and biological function are not clarified yet (Bleif et al. 2011, 2012; Schmitz et al. 2012; Kiss et al. 2015a, b). Recently, it was found that CYP106A2 is not strictly 15β-selective to progesterone, as positions 6β, 9α and 11α have also been observed as targets for hydroxylation particularly when adrenodoxin (Adx) and adrenodoxin reductase (AdR) or putidaredoxin reductase (PdR) and putidaredoxin (Pdx) served as redox systems (Fig. 8) (Lisurek et al. 2004, 2008; Nguyen et al. 2012; Virus et al. 2006).

Engineered *E. coli* or *B. subtilis* cells expressing CYP106A2 have been employed as biocatalysts for industrial biotransformations (Rauschenbach et al. 1993). In recent decades, the substrate spectrum of CYP106A2 has been largely expanded, demonstrating its excellent application in the pharmaceutical industry (Lisurek et al. 2004). Moreover, electron supply of CYP106A2 could be achieved with various redox systems, though its natural electron partners have never been identified (Ringle et al. 2013; Hannemann and Bernhardt 2006; Bleif et al. 2012; Schmitz et al. 2012). To further exploit the potential of this CYP, both rational mutagenesis and CAST have been applied to change the regio- and stereoselectivity towards steroid hydroxylation (Bernhardt 2008).

The structure of CYP106A2 was not clear until 2008, but a structural model has been built based on CYP11B1 by applying computational tools. With this information, a series of active-site mutants were created and screened for the improvement of steroid-hydroxylating activity (Lisurek et al. 2008). Mutants S394I, A395L, T396, G397P and Q398S exhibited decreased activity, especially for those mutants with mutations at the substrate recognition sites (A395, T396, G397). In terms of the selectivity and compared to the WT enzyme, mutant A395L showed 4- and 5-fold improvement for 11α- and 9α-hydroxylation of progesterone, respectively. Mutant G397P also showed more than four and twofold increase in the relative formation of 11α-hydroxyprogesterone and 6α-hydroxyprogesterone (Lisurek et al. 2008).

Subsequently, based on the homology model of CYP106A2 with the progesterone docked in the binding pocket, a CAST library at amino acid positions A395 and G397 lining in the binding pocket was created with the aim of switching the selectivity from C15 to C11 in progesterone hydroxylation (Nguyen et al.

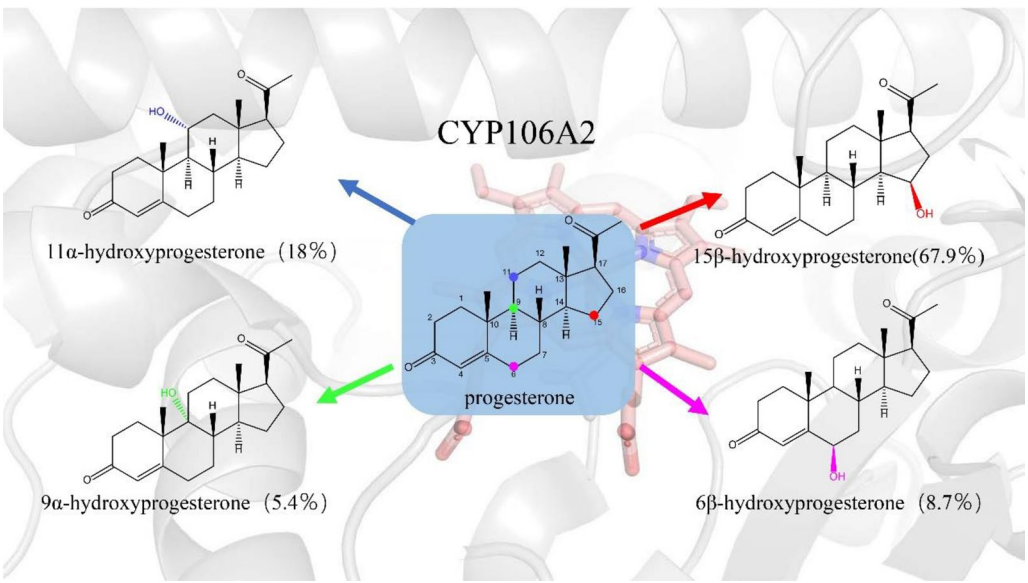

Fig. 8 Schematic representation of WT CYP106A2 (PDB: 4YT3) catalyzed hydroxylation of progesterone to four monohydroxylated products

2012). After screening the SM library, two mutants A395I and A395W/G397K were obtained with 8.9- and 11.5-fold enhancement for 11α-hydroxylase activity, respectively. To further increase catalytic efficiency, rational site-directed mutagenesis was undertaken to generate mutants A106T/A395I, A106T/A395I/R409L and T89N/A395I, which led to increased 11α-selectivity and activities relative to the WT (enhancements of 14, 13-, and 12-fold selectivity and 39, 108, and 24-fold K_{cat}/k_m) (Nguyen et al. 2012). The best mutant T89N/A395I was then employed in a whole-cell biotransformation with progesterone, indicating that the formation of 11α-hydroxyprogesterone increased from 28 to 81%, while 15β-hydroxyprogesterone decreased from 50.4 (WT) to 4.8% (Nguyen et al. 2012) (Fig. 9).

In 2016, CYP106A2 was crystallized and the 3D structure was solved (Janocha et al. 2016). With the structural information in hand, Nikolaus et al. tried to improve its 9α-hydroxylase and 6β-hydroxylase selectivity as well as activity (Nikolaus et al. 2017). Site-saturation mutagenesis was performed by targeting A395 and G397 to create a small library using CAST. Upon screening more than 13,000 clones with progesterone as model substrate, 16 mutants were obtained with improved 9α- and 6β-hydroxylase activity, and four of them exerted hydroxylation mainly at position C9α. To improve the variants, additional mutagenesis was performed and resulting mutant F165L/A395E/G397V exhibited 11-fold increase in selectivity towards progesterone 9α-hydroxylation. Meanwhile, mutant A243S was also discovered with improved selectivity towards 6β-hydroxyprogesterone from 9 to 86% (Nikolaus et al. 2017) (Table 2). Doubtless, CYP106A2 has been engineered with success using both rational design and directed evolution for some hydroxylation of pharmaceutical interest, which include positions 7α, 9α, 11α, 11β, 16α and 17α, among others (Donova and Egorova 2012).

CYP260A1: cytochrome P450 from myxobacterium *Sorangium cellulosum Soce56*

This enzyme was identified as a new class of bacterial P450 (Ewen et al. 2009; Khatri et al. 2016a) with a steroid hydroxylase activity towards C19 (testosterone and androstenedione) or C21 (11-deoxycorticosterone) type steroids at the very unique 1α-position in the presence of surrogate redox partners (adrenodoxin and adrenodoxin reductase) (Khatri et al. 2016b; Ewen et al. 2009; Schifrin et al. 2015; Litzenburger et al. 2015). However, the conversion of progesterone by CYP260A1 is very unselective (Khatri et al. 2016a, b; Salamanca-Pinzon et al. 2016), yielding a mixture of 1α-progesterone, 17α-progesterone and other products. Structure-guided rational design was conducted to mutate S326 to an asparagine, resulting in mutant S326R that displayed much better activity and selectivity for the formation of

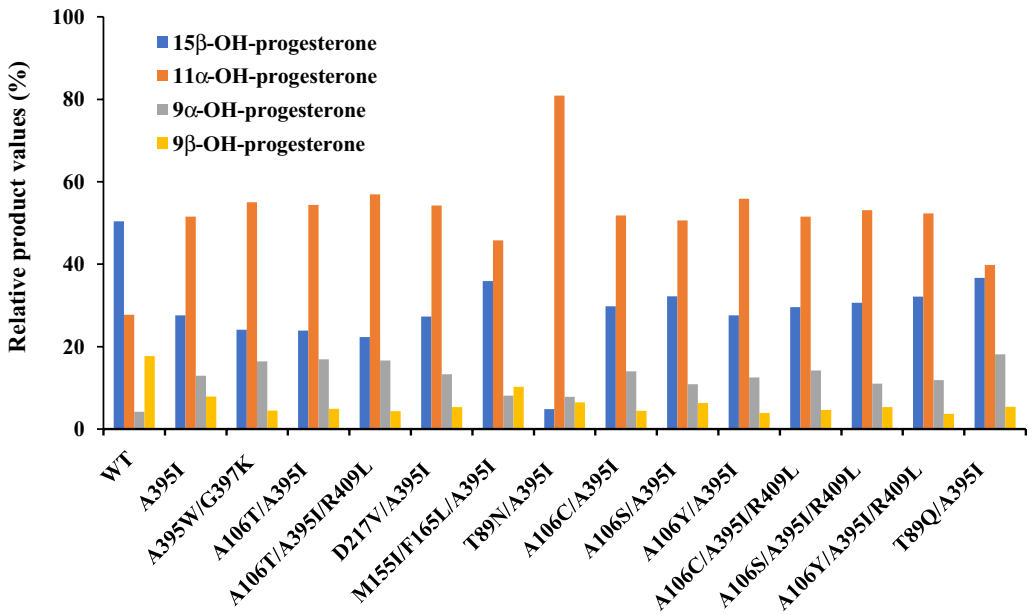

Fig. 9 Best CYP106A2 mutants on progesterone. Relative values [%] of progesterone substrate and monohydroxylated products (15β-, 11α-, 9α- and 6β) produced by CYP106A2 WT mutants A395I and G395W/G397K, and best combinatorially derived mutants in 6 h whole-cell biotransformations with 200 μM of progesterone (Nguyen et al. 2012)

Table 2 Selection of CYP106A2 mutants with different profiles for progesterone hydroxylation

Number	Mutation	15β [%]	11α [%]	9α [%]	6β [%]	Progesterone
1	WT-CYP106A2	67.9	18.0	5.4	8.7	n.d.*
2	A395T/G397K	11.2	20.0	45.1	23.7	n.d.*
3	A395H/G397Y	22.9	15.2	38.6	23.3	n.d.*
4	A395I	28.7	50.9	10.2	10.2	n.d.*
5	A395E/G397V	9.4	15.9	48.6	26.1	n.d.*
6	A395R/G397K	9.1	12.9	44.8	33.2	n.d.*
7	A395M/G397S	13.1	65.6	14.4	6.9	n.d.*
8	A395R/G397I	13.2	12.7	47.0	27.2	n.d.*
9	A395K	16.5	37.2	42.4	4.0	n.d.*
10	G397W	27.3	36.4	27.3	9.1	n.d.*
11	A395F	32.5	38.3	11.5	17.7	n.d.*
12	A243V	78.0	8.2	3.6	10.2	n.d.*
13	A395W/G397K	10.5	19.2	55.9	14.3	n.d.*
14	A395E/G397Stop	13.4	15.5	45.4	25.8	n.d.*
15	A395S/G397Y	18.5	13.7	41.1	26.8	n.d.*
16	A395R/G397R	8.8	14.9	45.8	30.6	n.d.*
17	D217V/A395E/G397V	7.5	23.2	55.9	7.9	5.4
18	F165L/A395E/G397V	11.3	17.2	59.7	11.0	0.9
19	A106T/A395R/G397K	13.0	22.4	57.4	6.8	0.4
20	A243V/A395R/G397R	11.8	17.6	49.7	8.3	12.7
21	A106T/A395R/G397R	12.9	14.9	56.2	13.3	2.7
22	F173A	9.7	32.5	44.1	5.0	8.6
23	A243S	3.4	4.6	2.4	82.7	6.9
24	T247A	11.7	13.1	45.7	24.3	4.3
25	A243S/T247A	14.6	13.2	48.0	20.7	20.7
26	F173A/A243S/T247A	13.6	6.5	8.7	57.2	19.1

Relative values in % of progesterone and its monohydroxylated products 15β-, 11α-, 9α- and 6β-hydroxyprogesterone after 24 h in vivo conversion of 200 μM progesterone catalyzed by WT CYP106A2 and mutant proteins thereof. Values show percentages with the sum of the substrate progesterone and all monohydroxylated products set as 100% (n.d.*: not detectable) (Nikolaus et al. 2017)

1α-hydroxy-11-deoxycorticosterone compared to the WT parent (Khatri et al. 2018).

The application of CYP260A1 for steroid conversion is often hindered because of its poor activity and low selectivity. In order to improve its regio- and stereoselectivity for progesterone transformation, Khatri et al. cloned and expressed truncated protein of CYP260A1 (ΔCYP260A1) (Khatri et al. 2018), and the first 50 N-terminal residues were removed based on the alternative predicted gene transcript from the NCBI database (Khatri et al. 2016a, b). Laborious efforts combining rational design and directed evolution were employed to create mutants of ΔCYP260A1 with improved regioselectivity towards progesterone hydroxylation (Khatri et al. 2018). After recovery of complete activity of ΔCYP260A1, the substrate was docked into ΔCYP260A1 active site to explore potential hotspot residues. The docking results suggested that three small polar residues (Ser225, Ser275 and Ser276) located at opposite sides of the binding pocket may interact with polar groups of progesterone during catalysis. Accordingly, a SM library based on CAST was created on those three residues by randomizing with NDC codon degeneracy (including 12 amino acid residues: Leu, Ile, Arg, His, Asp, Phe, Asn, Cys, Gly, Tyr, Val and Ser) using ΔCYP260A1 as template. Mutants of S225X and S275X were either inactive or maintained the similar catalytic activity with WT, and there were only five positive variants at position S276 (S276N, S276I, S276C, S276L and S276V) displaying an altered product profile relative to the WT ΔCYP260A1. Among them, mutants S276N and S276C displayed increased selectivity for 1α-hydroxyprogesterone (57 and 48 vs 36% of WT), with the former showing lesser formation of side products. In contrast, mutants S276I, S276L and S276V displayed moderately enhanced selectivity for product 17α-hydroxyprogesterone compared to the WT enzyme (64, 62 and 58 vs 39%, respectively) (Fig. 10)

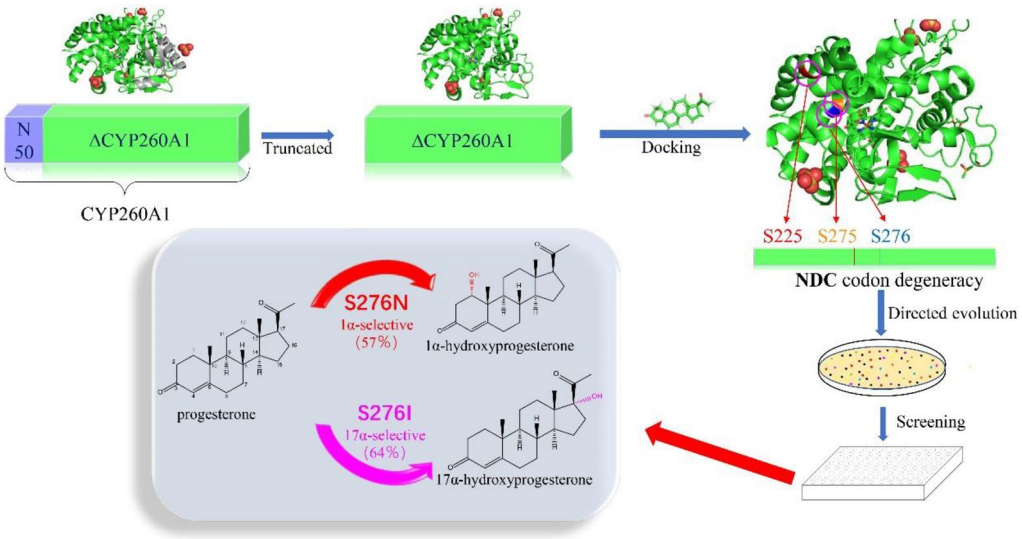

Fig. 10 Engineering CYP260A1 for progesterone functionalization at positions 1α and 17α. Strategy for constructing mutants of CYP260A1 and experimental workflow (Khatri et al. 2018)

(Khatri et al. 2018). With the addition of position 17α to the above targeted positions, it is clear that directed evolution can generate highly regio- and stereoselective enzymes for challenging biotransformation.

CYP260B1: cytochrome P450 from *Sorangium cellulosum*

CYP260B1 is a newly isolated CYP from *Sorangium cellulosum* with 48% homology to CYP260A1. Thus far, it has not been well characterized yet (Salamanca-Pinzon et al. 2016). Bernhart and coworkers used CYP260B1 to convert a small substrate library of six Δ^4-C21-steroids (progesterone, 17-hydroxyprogesterone, 11-deoxycorticosterone, cortisone, cortisol, and cortodoxone) and five steroidal drugs (dexamethasone, betamethasone, fluorine, budesonide, and desonide) with the adrenal redox partners (Adx and AdR) (Litzenburger and Bernhardt 2017). CYP260B1 showed activity towards most substrates tested except for the steroidal drugs with a fused heterocyclic ring (such as fluorine, budesonide, and desonide), with the highest activity and selectivity achieved for 11-deoxycorticosterone and cortodoxone. For the transformation of 11-deoxycorticosterone, conversion reached almost 100% and the major product was 9α-OH-11-deoxycorticosterone with 62% selectivity. In the case of cortodoxone, conversion reached 95% and the major product was 6β-OH-cortodoxone with 94% selectivity. The mutant CYP260B1-T224A was created and tested (Litzenburger and Bernhardt 2017), enabling an increase from 62 to 75% 9α-hydroxy-11-deoxycorticosterone selectivity without activity decline (Fig. 11). The synthetic potential of CYP260B1 remains to be explored.

Conclusions

In this review, we summarized the achievements attained in the engineering of bacterial CYPs towards steroid hydroxylation during the past decades. Since most WT P450s often suffer from wrong or insufficient enantio- and/or regioselectivity, low activity, narrow substrate range, and insufficient thermostability, laboratory evolution and rational design have emerged as promising tools to solve these problems. Relevant examples have been summarized in the synthesis of valuable hydroxylated steroid compounds with different types of bacterial P450s. Notably, while some studies have developed novel directed evolution strategies, others have shown that many P450s still await discovery and characterization. We believe that a combination of both strategies will allow the controlled regio- and stereoselective hydroxylation at any position of interest of a wide range of steroids for the development of more sustainable, efficient and economic biotechnological processes.

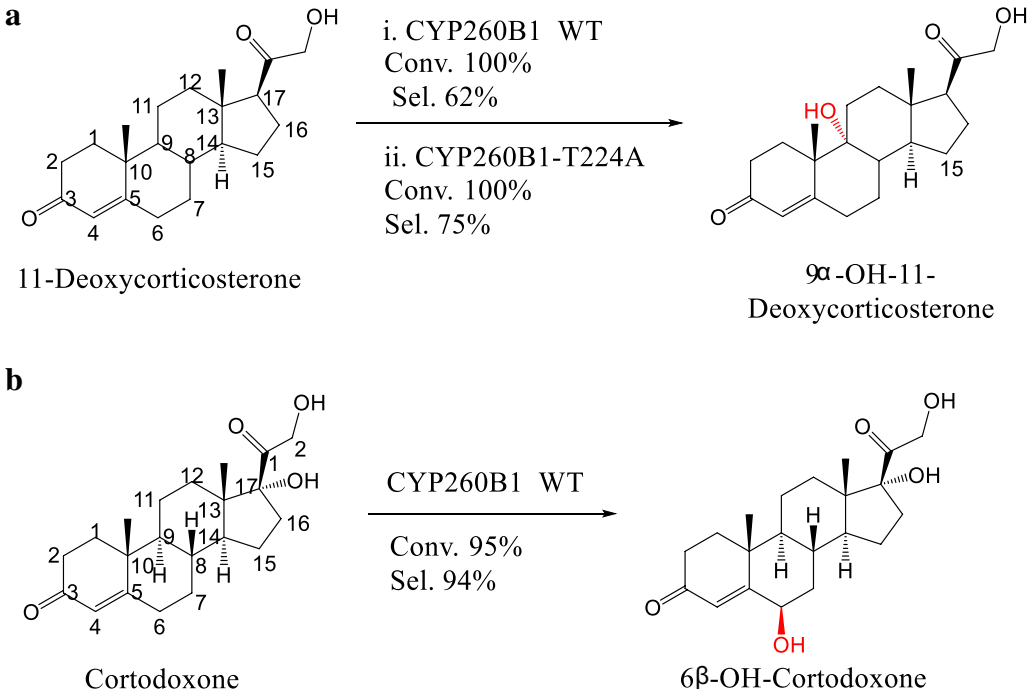

Fig. 11 The conversion and major hydroxylated products of 11-deoxycorticosterone (**a**) and cortodoxone (**b**) catalyzed by CYP260B1 and CYP260B1-T224A. Reaction conditions: purified enzymes of CYP260B1 and its T224A (0.5 μM), AdR (1.5 μM), Adx4–108 (10 μM), $MgCl_2$ (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1 U) in a final volume of 250 μl of potassium phosphate buffer (20 mM, pH 7.4) was used. The steroids (10 mM stock solution in EtOH) were added to a final concentration of 200 μM. The reactions were initiated by adding 500 μM NADPH, 30 min, 30 °C

Abbreviations

CYPs: cytochrome P450 monooxygenases; WT: wild type; FAD: flavin adenine dinucleotide; FMN: flavin mononucleotide; FeS: iron–sulfur cluster; NADH: nicotinamide adenine dinucleotide; NADPH: nicotinamide adenine dinucleotide phosphate; SRS: substrate recognition sites; epPCR: error-prone PCR; SM: saturation mutagenesis; ISM: iterative saturation mutagenesis; CAST: combinatorial active-site saturation test; MLs: mutability landscapes; MDs: molecular dynamics simulation; Adx: adrenodoxin; AdR: adrenodoxin reductase; PdR: putidaredoxin reductase; Pdx: putidaredoxin.

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Authors' contributions

AL conceived and provided advice in this review. XZ and YP were involved in collecting related material and drafted the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Ethics approval and consent to participate

All the authors have read and agreed the ethics for publishing the manuscript.

Consent for publication

The authors approved the consent for publishing the manuscript.

Competing interests

The authors declare that they have no competing interests.

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