Engineered P450 Enzymes for Specific Oxidative Modification of Steroid Compounds (Postprint)

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Abstract

Steroid drugs constitute the second largest class of pharmaceuticals worldwide after antibiotics, exhibiting important therapeutic effects such as anti-inflammatory, anti-allergic, and endocrine-modulating activities. Effective oxidative modification at specific sites of the steroid nucleus is pivotal for introducing pharmacological activity. Studies have shown that P450 enzymes represent a key enzyme family that catalyzes the site-specific oxidation of steroids. Currently, electron transfer efficiency and catalytic specificity are critical factors limiting the catalytic performance of P450 enzymes, leading to problems including low yields of target steroid products and severe accumulation of by-products. Therefore, this article will systematically review the methodological strategies and related research progress in engineering P450-catalyzed steroid transformations, focusing on research efforts to improve catalytic efficiency and specificity. Additionally, it will provide an outlook on the future development of P450 enzyme design and optimization for steroid compound catalysis, offering guidance for in-depth investigation in this area.

Full Text

Preamble

Engineering P450 for Specific Oxidation of Steroids

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Abstract

Steroidal drugs represent the second largest class of pharmaceuticals globally, after antibiotics, and possess important therapeutic activities including anti-inflammatory, anti-allergic, and endocrine-regulating effects. Effective and specific oxidative modification of the steroid nucleus at particular positions is the key step for introducing pharmacological activity. Research has shown that cytochrome P450 enzymes are a critical enzyme family that catalyzes the specific oxidation of steroids. Currently, electron transfer efficiency and catalytic specificity are important factors limiting P450 catalytic function, leading to low yields of target steroid products and severe accumulation of by-products. Therefore, this review systematically summarizes the methodological strategies and research progress in engineering P450 enzymes for catalyzing steroid compounds, focusing on improving catalytic efficiency and specificity. We also provide perspectives on future directions for the design and optimization of P450 enzymes that catalyze steroid oxidation, offering guidance for in-depth research in this field.

Keywords: Steroids; P450 monooxygenase; Electron transfer; Catalytic specificity

Introduction

Steroidal drugs are the second largest class of pharmaceuticals worldwide, with over 400 different steroid drugs currently in production and global sales reaching $100 billion by 2017. Due to their significant regulatory functions in immune response, sexual function, glucose metabolism, and lipid metabolism, steroid compounds are widely used to treat various inflammatory conditions, allergic reactions, and diseases affecting the respiratory, cardiovascular, endocrine, and tumor systems. As a major producer of steroid hormone drug raw materials and formulations, China has prioritized the development of new steroid drug resources as a key direction for the pharmaceutical industry' s near-term growth.

In the synthesis of steroid drugs, specific oxidative modification of the steroid nucleus is a crucial catalytic step for introducing pharmacological activity. This step typically involves oxidation at positions such as C1, C9, C11, C16, C17, and C19 on the steroid ring, with catalytic types including C-C bond cleavage and oxidative modifications represented by hydroxylation reactions (Figure 1 [Figure 1: see original paper]). Hydroxylation modification can increase drug polarity, thereby improving steroid drug solubility and blood concentration, while also activating the substrate to facilitate subsequent catalytic reactions such as glycosylation. Therefore, hydroxylation is critical for steroid drug activity.

Currently, steroid oxidation reactions are achieved primarily through chemical modification or microbial transformation. Compared with traditional chemical catalysis, biocatalysis offers lower energy consumption and greater environmental friendliness, while also demonstrating superior catalytic specificity under certain conditions. Consequently, an increasing number of researchers aim to

leverage the high efficiency and product specificity advantages of biocatalysis to achieve oxidative modification of the steroid nucleus, investigating and optimizing steroid transformation systems in vivo and gradually introducing key enzyme systems into heterologous model organisms to construct efficient steroid transformation systems.

Research indicates that the vast majority of steroid modifications in organisms are mediated by cytochrome P450 monooxygenases (Figure 1), which represent the most important enzyme system for catalyzing steroid oxidation reactions. These enzymes use the cofactor NAD(P)H as an electron donor and utilize the oxidizing capacity of heme iron to achieve targeted monooxygenation reactions. However, researchers often cannot achieve ideal steroid oxidation effects through simple expression of natural P450 monooxygenases. This is because P450 proteins achieve membrane localization through a conserved hydrophobic domain at their N-terminus. Additionally, due to differences in intracellular microenvironment and protein expression systems between heterologous microbial hosts and natural hosts, heterologously expressed P450 monooxygenases often exhibit low expression levels and catalytic efficiency due to improper protein folding or subcellular localization.

Furthermore, P450 monooxygenases require electron transfer system assistance to effectively transfer electrons from NAD(P)H to the catalytic center' s heme, completing the oxidation of steroid substrates. The interaction between the protein and the host' s endogenous or heterologously expressed electron transfer system directly determines electron transfer efficiency, thereby affecting P450 monooxygenase catalytic efficiency. Combined with the catalytic characteristics of P450 proteins and the high structural similarity of steroid substrates, certain P450 monooxygenases exhibit poor specificity when catalyzing steroid substrates. Therefore, developing engineering strategies for P450 monooxygenases and their electron transfer systems is of significant research importance and application value for improving oxidative efficiency and product specificity in steroid modification.

Current strategies to address P450 monooxygenase expression issues (Figure 2 [Figure 2: see original paper], white sections) include: (1) modifying the N-terminus of P450 enzymes to improve effective localization and soluble expression levels in heterologous hosts; (2) assisting correct folding of P450 monooxygenases through chaperone co-expression to ensure catalytic activity; and (3) improving P450 monooxygenase expression levels and catalytic efficiency through site-directed mutagenesis. Since these engineering methods have been systematically reviewed by Hausjell et al. and Susan et al., they will not be reiterated here. This review focuses on summarizing research efforts to enhance catalytic reaction efficiency by strengthening electron transfer efficiency and to improve catalytic specificity by optimizing key structures of P450 monooxygenases (Figure 2, orange sections). We describe in detail how engineered heterologous P450 catalytic systems can achieve efficient and specific oxidative modification of the steroid nucleus in model microbial hosts, providing reference guidance for re-

lated research.

1. Engineering P450 Catalytic Efficiency for Steroids Based on Electron Transfer Efficiency

P450 catalytic function depends on specific electron transfer processes between oxidases and reductases. In vivo, P450 enzymes are divided into two major classes (Class I and Class II). Class I P450 enzymes localize to the inner membrane systems of prokaryotes or the mitochondrial inner membrane of eukaryotes, while Class II P450 proteins localize to the endoplasmic reticulum membrane of eukaryotes. These two classes of P450 enzyme complexes contain different subunits and distinct electron transfer systems (Figure 3 [Figure 3: see original paper]A). The Class I P450 electron transfer system consists of ferredoxin (Fdx) containing iron-sulfur clusters and its paired flavoprotein ferredoxin reductase (FdR) containing FAD cofactors, or alternatively flavodoxin (Fld) and its paired flavodoxin reductase (FpR). The most widely applied system is adrenodoxin (Adx) and adrenodoxin reductase (AdR) derived from mammalian mitochondria. The Adx/AdR electron transfer system can support not only mitochondrial P450 enzymes from various eukaryotic sources but also interact with some prokaryotic P450 oxidases to support their catalyzed oxidation reactions.

The Class II P450 electron transfer system contains only the NADPH-cytochrome P450 reductase (CPR) with FAD and FMN prosthetic groups (Figure 3A). Both the oxidase and reductase of Class II P450 are anchored to the endoplasmic reticulum membrane through N-terminal hydrophobic sequences, with their catalytic domains oriented toward the cytoplasmic side. Electron transfer occurs through random molecular collisions between the two components.

During heterologous expression of P450 catalytic systems, low expression levels, poor activity, or difficult synergistic interaction between oxidases and reductases often result in low electron transfer efficiency, greatly inhibiting P450 oxidase catalytic efficiency. To address these issues, researchers typically employ the following strategies (Figure 3): (1) matching P450 oxidases with highly coupled redox partners; (2) supplementing redox power by adjusting the ratio of redox enzymes and their partners or expressing additional electron transfer components; and (3) artificial assembly of P450 oxidases with their redox partners. Here, we elaborate on practical problems encountered during heterologous construction of P450 catalytic systems for oxidizing steroid nuclei.

1.1 Matching P450 Oxidases with Redox Partners

Co-expressing P450 oxidases with redox partners that have high coupling efficiency in the host is an effective means to improve electron transfer efficiency in P450 catalytic systems. To achieve this goal, researchers often select redox partners from different sources for combinatorial screening with target P450 oxidases (Figure 3B). Screening typically begins with selecting electron transfer systems

belonging to the same class as the P450 oxidase. For Class I P450 oxidases, commonly used systems besides the most common Adx/AdR include putidaredoxin (Pdx)/putidaredoxin reductase (PdR) from *Pseudomonas putida*, spinach ferredoxin Fdx/reductase FdR from spinach mitochondria, and Etp1fd (ferredoxin domain of electron transfer protein 1)/Arh1 (adrenodoxin reductase homologue 1) from fission yeast (*Schizosaccharomyces pombe*), where Etp1fd/Arh1 is highly homologous to Adx/AdR.

Based on successful cases of improved steroid ring oxidation efficiency listed in Table 1 , we find that redox partners with high coupling efficiency to heterologous P450 oxidases in hosts are generally not their naturally matched electron transfer systems. For example, co-expression of CYP260B1 from *Sorangium cellulosum*, bovine Adx, and *E. coli* FpR in *Escherichia coli* achieved the highest hydroxylation efficiency at the C9 position of 11-deoxycorticosterone. In fission yeast, when human P450 oxidases were co-expressed with reductases from *Ammi majus*, human, and endogenous fission yeast sources, human CYP17 and CYP21 showed highest catalytic activity when matched with endogenous fission yeast CPR. This may be because isozymes from different species inherently have large activity differences, and host cell compatibility also affects heterologous gene expression. Therefore, screening and optimizing combinations of electron transfer systems from different sources with P450 oxidases is an effective means to improve electron transfer efficiency.

For instance, when expressing CYP154C5 from *Nocardia farcinica* in *E. coli*, co-expression of Pdx/PdR from *Pseudomonas putida* enabled the conversion of pregnenolone to 16 -hydroxypregnenolone. Interestingly, in some cases, P450 oxidases can accept electrons from electron transfer systems belonging to a different class. For example, when a Class I P450 oxidase is localized to the endoplasmic reticulum in yeast, it can accept electrons from CPR belonging to the Class II system. Toshiyuki et al. expressed rat mitochondrial CYP27 in *Saccharomyces cerevisiae* by replacing its N-terminal mitochondrial targeting sequence with an endoplasmic reticulum targeting sequence to achieve ER localization. Under the action of endogenous yeast CPR, electrons were successfully transferred to CYP27 to achieve C27 hydroxylation of 5 -cholestane-3 ,7 ,12 -triol.

Similarly, heterologously expressed Class II P450 oxidases can sometimes accept electrons from Class I electron transfer chains, even achieving optimal electron transfer efficiency. For example, when expressing mammalian endoplasmic reticulum-derived CYP21A2 in *E. coli*, co-expression of Etp1fd/Arh1 resulted in a 1.92-fold higher hydroxylation rate at the C21 position of medrol compared to co-expression with CPR. Therefore, the host' s endogenous environment may directly affect the matching degree between P450 oxidases and redox partners. Although the determining factors and mechanisms remain unclear, these cases remind us to consider heterologous electron transfer systems co-localized with P450 oxidases when matching them with redox partners. Further mechanistic analysis of these examples will help design and construct heterologous P450 catalytic systems for efficient steroid oxidation.

1.2 Supplementing Redox Power

As mentioned previously, initially expressed P450 oxidases and reductases often suffer from low heterologous expression levels and poor activity, affecting electron transfer efficiency. In such cases, redox power must be supplemented to enhance electron transfer rates and improve P450 oxidase catalytic capacity toward steroid substrates. Based on successful examples of steroid P450 systems listed in Table 1, two main approaches are currently employed: adjusting the expression ratio of protein subunits in the P450 redox enzyme system and expressing additional electron transfer components (Figure 3C). These strategies enhance electron transfer efficiency by regulating electron transfer flux.

1.2.1 Adjusting the Ratio of Oxidases and Their Redox Partners Regulating the expression ratio of protein subunits in the P450 enzyme system is a common method for controlling redox power (Figure 3C). For example, Sawada et al. expressed CYP105A1 from *Streptomyces griseolus* with spinach ferredoxin and reductase Fdx/FdR in *E. coli* to convert VD2 to 1 ,25-(OH)VD2. Their results showed that co-expression at 4 µM Fdx/0.2 µM FdR yielded much higher CYP105A1 catalytic activity than at 4 µM Fdx/2 µM FdR. Lina et al. enhanced CYP11B1 catalytic efficiency toward 11-deoxycortisol by increasing Adx copy number. They found that expressing three copies of Adx was more favorable for 11-deoxycortisol conversion than a single copy. When Adx expression increased 3.3-fold, hydrocortisone production increased by 30%. Thus, for Class I P450 redox systems, maintaining high electron transfer efficiency requires a high ferredoxin/ferredoxin reductase ratio, likely related to the bridging role of ferredoxin in Class I electron transfer chains.

1.2.2 Expressing Additional Electron Transfer Components Expressing additional electron transfer components is a common method for supplementing redox power, particularly when constructing Class II P450 catalytic systems, where researchers tend to additionally express cytochrome b5 (Cyt-b5) (Figure 3C). For example, Vimercati et al. heterologously expressed three equine CYP3A family P450 enzymes with CPR and Cyt-b5 from the same source at a 1:4:1 ratio in insect cells, achieving 6-position hydroxylation of testosterone. However, for certain P450-catalyzed steroid oxidation reactions, additional Cyt-b5 expression does not enhance electron transfer efficiency. For CYP17A1, which possesses both C17 hydroxylation and C17-C20 bond cleavage activities, additional Cyt-b5 expression only enhances the latter activity.

Ruchia et al. used nanodisc technology to demonstrate that Cyt-b5 can provide an additional electron during reduction of the oxyferrous complex to the peroxo-ferric intermediate, accelerating activation of reaction intermediates. In CYP17A1-catalyzed cleavage reactions, Cyt-b5 reduces the oxyferrous complex 10 times faster than CPR, and the electron transfer chain coupling rate is 5-fold higher with Cyt-b5 than without it. Although cytochrome b5 can effectively enhance electron transfer efficiency, Sang et al. reported that Cyt-b5 introduction

caused conformational changes in the CYP2B4 catalytic center when catalyzing methoxyflurane, with the extent of change depending on the CPR/Cyt-b5 ratio. Therefore, Cyt-b5 application in steroid oxidation reactions should be approached cautiously, with the expression ratio of Cyt-b5 to reductase adjusted when necessary.

1.3 Artificial Assembly of P450 Oxidases and Their Redox Partners

Both eukaryotic and prokaryotic organisms contain proteins expressed as Class II P450 oxidase-CPR fusions. These proteins utilize naturally fused electron transfer chains to achieve more efficient electron transfer, with rates superior to P450 isozymes where oxidase and redox partner are expressed separately. The typical representative is P450 BM3 (CYP102A1), whose fatty acid hydroxylation reaction rate is more than two orders of magnitude higher than eukaryotic fatty acid hydroxylases. Therefore, fusing P450 oxidases with their redox partners is a strategy to enhance electron transfer efficiency by shortening spatial distance (Figure 3D).

For example, in natural fusion proteins, the reductase component of P450 BM3 (CYP102A1) can match with various heme proteins. Sandra et al. fused CYP130 from *Mycobacterium tuberculosis* (with unknown natural redox partner) with the NADPH reductase domain of P450 BM3 (CYP102A1) from *Bacillus megaterium* using a linker. The fused enzyme showed 6% higher catalytic activity toward dextromethorphan than non-native redox partner-reconstituted CYP130. For Class I P450 catalytic systems, constructing triple fusion proteins of oxidase-ferredoxin-ferredoxin reductase or fusing ferredoxin-ferredoxin reductase as a single reductase unit also improves electron transfer efficiency. For example, Eachan et al. fused CYP101A1 from *Pseudomonas putida* with its homologous ferredoxin reductase (PdR), using free ferredoxin (PdX) as the electron transfer component, which increased the 5-hydroxylation efficiency of camphor to twice that of the natural system.

However, despite the effectiveness of fusion expression, recent attempts to fuse steroid oxidases with their redox partners have struggled to improve catalytic efficiency of steroid P450 monooxygenases. Charles et al. fused bovine CYP17A with rat P450 reductase to catalyze 17-hydroxylation of pregnenolone, but the fusion protein did not achieve improved catalytic efficiency compared to separate expression. Patrick et al. fused flavodoxin YkuN from *Bacillus subtilis* with flavodoxin reductase Fpr from *E. coli*, matching them with CYP106A2 from *B. megaterium* and bovine CYP21A2 for in vitro catalytic modification of progesterone. Although the YkuN/Fpr fusion system could improve coupling efficiency with CYP106A2 and CYP21A2, the reaction rate was only 35.1% and 50.0% of that when YkuN and Fpr were expressed separately.

Strushkevich et al. fused CYP11A1/Adx with three different linkers and found that the kcat of all fusion proteins was far lower than that of separately expressed CYP11A1. Structural analysis of the Adx/CYP11A1 complex revealed

that electron transfer depends on specific orientation-dependent interface interactions between Adx and CYP11A1. Therefore, fusing ferredoxin Adx with ferredoxin reductase AdR may hinder these specific interface interactions, preventing effective connection of the electron transfer chain between the Adx iron-sulfur cluster and CYP11A1 heme. These experimental results and structural analyses indicate that for Class I P450 systems containing ferredoxin, protein fusion strategies for steroid ring oxidation should focus on interface interaction-based electron transfer processes, with analysis of protein subunit interactions helping to improve fusion effectiveness. For example, proliferating cell nuclear antigens (PCNAs) from *Sulfolobus solfataricus* form a heterotrimer. Hidehiko et al. fused oxidase P450cam, PdX, and PdR each with one PCNA subunit, using PCNA self-assembly to guide interface interactions among P450cam, PdX, and PdR. The resulting non-covalent heterotrimeric complex showed highly efficient electron transfer rates, with NADPH and O2 consumption rates 50-fold higher than independently expressed P450cam, PdX, and PdR. Therefore, developing assembly strategies for P450 oxidases and their redox partners may be an effective solution to the current difficulty of achieving positive effects from fusion expression in steroid P450 catalytic systems.

2. Improving Catalytic Specificity of Engineered Steroid P450 Monooxygenases

Due to the high structural conservation of steroid nuclei and the similarity of functional groups undergoing oxidative modification (primarily methyl and methylene groups), most natural steroid P450 monooxygenases exhibit poor catalytic specificity. For example, CYP106A2 possesses hydroxylation activity at steroid ring positions C6, C9, and C15 simultaneously, while CYP27A1 can hydroxylate positions C25, C26, and C27. In practical applications, however, specific site oxidation of the steroid ring is required to exert unique functional activity of the catalytic product. To meet application requirements, target P450 proteins must be engineered and optimized to obtain engineered P450s with excellent catalytic specificity. Current main strategies for P450 modification and optimization are divided into semi-rational design and rational design, with irrational design being less commonly applied. These approaches are discussed separately below.

2.1 Irrational Design-Based Directed Evolution of Steroid P450 Monooxygenases

Irrational design of steroid P450s primarily involves random mutagenesis of target steroid P450 monooxygenase genes through error-prone PCR, followed by high-throughput screening to obtain mutants with desired catalytic effects. For example, wild-type P450 BM3 cannot catalyze 17 -estradiol. Cha et al. performed error-prone PCR on the P450 BM3 coding gene and identified the mutant R47L/E64G/F81I/F87V/E143G/L188Q/E267V in the random mutation library that could achieve C2 hydroxylation of 17 -estradiol when expressed in

E. coli.

However, few studies have reported optimization of steroid P450 monooxygenase catalytic specificity through irrational design, and the efficiency is low. This is because irrational design often focuses on improving expression levels and catalytic efficiency rather than specificity. For example, Schiffer et al. performed random mutagenesis on human CYP11B1 and screened the excellent mutant L271M, which showed highest conversion efficiency for catalyzing 11-deoxycortisol to hydrocortisone. Additionally, irrational design often requires high-throughput screening, but most steroid biosynthesis pathway intermediates lack color or special physicochemical properties (such as microbial resistance or functional group-specific color reactions), making high-throughput screening difficult. Due to these limitations, irrational design is rarely applied to optimize steroid P450 monooxygenase catalytic specificity. Therefore, researchers typically employ semi-rational or rational design approaches to achieve this goal.

2.2 Semi-Rational Design-Based Directed Evolution of Steroid P450 Monooxygenases

Many steroid P450 monooxygenase structures and their complexes with substrate analogs have been reported. Combined with the structural conservation of steroid P450 monooxygenases and advances in structural simulation methods, these provide a rich and reliable structural basis for P450 monooxygenase research and modification. Semi-rational design analyzes these complex structures to predict several sites affecting P450 monooxygenase catalytic selectivity, followed by saturation mutagenesis to enhance specific oxidation at target positions on the steroid ring.

For example, CYP260A1 from *Sorangium cellulosum* specifically catalyzes C1 hydroxylation of C19-steroids (e.g., androsterone, androstenedione) and C21-steroids (e.g., 11-deoxycorticosterone), but primarily hydroxylates progesterone at C3 and C5 positions in vitro. To enhance CYP260A1 selectivity for the C1 position, Khatri et al. docked the crystal structure of N-terminally truncated CYP260A1 with progesterone to simulate the protein-substrate complex structure, initially identifying amino acid residues S225, S275, and S276 as directly related to CYP260A1 catalytic selectivity toward progesterone. Saturation mutagenesis at these three positions revealed that mutant S276N increased the proportion of 1 -hydroxy-progesterone in total products from 36% to 57%. Compared with irrational design, semi-rational design reduces randomness in target selection, decreases the size of mutant libraries requiring screening, and avoids substantial consumption of resources.

2.3 Rational Design-Based Directed Evolution of Steroid P450 Monooxygenases

Rational design is based on thorough understanding of steroid P450 monooxygenase protein structures and catalytic mechanisms, enabling precise regulation of

target protein structures to alter catalytic selectivity. In addition to analyzing P450 protein-steroid substrate complex structures, rational design can identify potential specificity-determining sites through homology sequence alignment between isozymes with different catalytic selectivities. For example, in studying CYP17A1 substrate selectivity for C17-C20 bond cleavage, Gregory et al. performed homology sequence alignment of CYP17A1 from eight different vertebrate sources, finding that when residue 202 was asparagine, CYP17A1 tended to catalyze substrates with hydroxyl groups at the steroid ring C3 position (e.g., 17-hydroxypregnenolone), while when residue 202 was serine or threonine, it preferred substrates with keto groups at C3 (e.g., 17-hydroxyprogesterone). Therefore, they proposed that residue 202 determines CYP17A1 substrate selectivity, and the human CYP17A1 mutant N202S reversed the optimal substrate from 17-hydroxypregnenolone to 17-hydroxyprogesterone.

Swart et al. analyzed sequences of CYP17A1 from 18 different sources, finding that CYP17A1 with strong C16 hydroxylation capability typically had alanine at position 105, while those with weak C16 hydroxylation had leucine at this position. Expressing human CYP17A1 A105L in mammalian COS-1 cells for progesterone biotransformation increased the 17-hydroxyprogesterone to 16-hydroxyprogesterone product ratio from 4:1 (wild-type) to 9:1. Therefore, selecting isozymes with different catalytic specificities for homology sequence alignment and mutating conserved sites of one specificity class to those of another is a simple and effective strategy for altering steroid P450 catalytic selectivity without requiring protein complex structural knowledge.

As shown in Table 2 , key amino acid residues determining steroid P450 catalytic selectivity are mostly located at or near the catalytic active center. Mutations at these sites aim to fine-tune substrate recognition sites or substrate access channels without compromising catalytic activity, adjusting target substrate binding capacity or spatial orientation in the active center pocket to alter the distance between target sites on the steroid ring and the active center heme, thereby obtaining desired catalytic selectivity for practical applications.

Based on cases listed in Table 2, strategies for altering catalytic selectivity can be divided into three categories (Figure 4 [Figure 4: see original paper]):

(1) Adjusting Spatial Hindrance Between Substrate and Active Center Heme

As shown in Table 3 , successful cases of altering steroid P450 monooxygenase catalytic specificity in the past decade have mostly occurred in the A, C, and D rings of the steroid nucleus, rarely in the B ring. This may be due to greater spatial hindrance between the steroid B ring and the heme. This suggests that by changing the length of amino acid side chains at specific positions, we can alter the relative position between the substrate catalytic site and the P450 active center heme through spatial hindrance adjustment, thereby changing target protein catalytic selectivity. For example, mutating human CYP17A1 residue 105 from alanine (smallest side chain) to leucine (longer side chain) increases spatial

hindrance between progesterone C16 and CYP17A1 heme, increasing catalytic difficulty at progesterone C16 and reducing by-product 16-hydroxyprogesterone formation.

Paolo et al. sequentially mutated amino acid residues with small nonpolar side chains in the CYP3A4 active center to tryptophan or phenylalanine with larger nonpolar side chains, following the order of distance from the heme (near to far), to reduce progesterone binding affinity. While wild-type CYP3A4 hydroxylated progesterone to produce large amounts of 6 -hydroxyprogesterone and small amounts of 2 -hydroxyprogesterone plus other by-products, mutant I301F/I369F/L482F showed balanced hydroxylation capacity at progesterone C-2 and C-6 positions, with other by-products reduced by half. The large side chain rigid topology structure reduced ligand binding freedom, thereby limiting specific catalytic sites.

(2) Adjusting Relative Position Between Substrate and Active Center Heme

Mutating key site amino acid residues to those containing hydroxyl groups or larger hydrophobic side chains can adjust the relative position between the heme iron center and target catalytic sites through hydrogen bonding or hydrophobic interactions. For example, for C11 hydroxylation of 17-hydroxyprogesterone in *E. coli*, Xiong et al. mutated human CYP11B1 L382 to serine (containing hydroxyl side chain). The L382S mutation could form new hydrogen bonds with heme carboxyl groups, shifting the heme toward the substrate C11 position. Simultaneously, the flexibility from the nearby F381A mutation enhanced the interaction between L382S and heme (Figure 4B). Compared with wild-type CYP11B1, mutant F381A/L382S increased the proportion of 21-deoxycortisol in total products from 26.5% to 53.5%, with conversion rate increasing by 39.1%. Thus, adjusting the relative position between the substrate catalytic site and active center heme can not only enhance target oxidation specificity but also improve steroid P450 monooxygenase catalytic efficiency.

(3) Adjusting Substrate Spatial Orientation in the Catalytic Active Center

Selecting amino acid residues related to substrate binding for mutation can alter steroid substrate spatial orientation in the P450 active center by enhancing or weakening hydrogen bonding or hydrophobic interactions between the residue and substrate, thereby adjusting the distance between target sites and heme to regulate P450 monooxygenase catalytic selectivity. For example, Khatri et al. found that progesterone could bind to the CYP260A1 catalytic active center in two different spatial orientations. When progesterone C3- and C20-keto groups formed hydrogen bond interactions with S225 and S276 of N-terminally truncated CYP260A1 (tCYP260A1), the substrate C1 position was near the heme and CYP260A1 preferentially catalyzed progesterone C1 (spatial position 1). When progesterone C3- and C20-keto groups interacted with S276 and S225 of tCYP260A1, the substrate molecule' s relative spatial position in the active

center was reversed, with C17 near the heme and CYP260A1 preferentially catalyzed progesterone C17 (spatial position 2).

CYP260A1 mutant S276N disrupted the hydrogen bond between wild-type S276 and progesterone C3-keto group, preventing progesterone from stably binding in spatial position 2, so the mutant produced mainly 1-hydroxyprogesterone. Conversely, mutant S276I did not affect progesterone binding in spatial position 2 (the I276 mutation could form hydrophobic interactions with the substrate A ring to compensate for hydrogen bond loss at this site), but the larger hydrophobic side chain of I276 hindered formation of the original hydrogen bond with the substrate C20-keto group, preventing progesterone from stably binding in spatial position 1, so this mutant produced mainly 17-hydroxyprogesterone.

Conclusion and Perspectives

Steroid biotransformation research has nearly 70 years of history, providing mature understanding of steroid biosynthetic pathways and key enzymes, especially cytochrome P450 monooxygenases. With scientific and technological advances, engineering P450 monooxygenases can effectively improve the efficiency and selectivity of steroid oxidative modification. Current research has found that in natural P450 electron transfer chains, electron utilization is limited by electron energy and supply, often failing to meet catalytic reaction energy demands. Introducing new electron supply systems, such as H2O2-driven or light-driven electron donors, promises to break through the ceiling of natural P450 electron transfer while facilitating regulation of electron supply balance. Additionally, new electron supply systems can be applied in P450-catalyzed in vitro reactions to reduce or avoid consumption of expensive NAD(P)H cofactors. Although these methods have not been widely applied in P450 monooxygenase-catalyzed steroid reactions, this direction may bring entirely new breakthroughs to steroid oxidative modification.

Furthermore, three important technical issues remain in practical steroid production: low substrate solubility, poor modification specificity, and high product concentration inhibition. These can be addressed through existing research approaches:

(1) Low Steroid Solubility: Steroid compounds have extremely low solubility in aqueous media, requiring organic solvents such as methanol, polyethylene glycol 400, cyclodextrin, or propylene glycol as cosolvents to increase available substrate concentration. However, cosolvent addition may damage host cell membranes and cause P450 protein denaturation. For membrane damage, expression of related genes (e.g., lipid metabolism genes) can be regulated to improve membrane stability. For enzyme denaturation, protein mutagenesis and recombination can obtain solvent-tolerant protein mutants. Additionally, with the realization of de novo synthesis of steroid drug intermediates such as progesterone and hydrocortisone, synthetic biology is promoting a transition in steroid drug production from initial biotransformation to "synthesis from

simple carbon sources using artificially engineered functional microorganisms." This de novo synthesis mode can also avoid the problem of low available steroid substrate concentrations.

(2) Poor Modification Specificity: Constructing heterologous steroid expression pathways involves multi-step sequential P450-catalyzed reactions. In actual pathways, non-specific substrate catalysis creates complex network catalytic pathways, making P450 catalytic selectivity issues more prominent and posing enormous challenges for P450 mutagenesis design. As described previously, we have systematically detailed how rational, semi-rational, and irrational design can mutate amino acid residues to solve specificity problems in steroid production. However, for network synthesis pathways, it is difficult to rely solely on directed evolution of relevant P450 proteins. Fortunately, research has found that enzymes in natural sterol or steroid synthesis pathways in eukaryotic cells are often localized to different organelles, providing excellent basis for compartmentalized reaction design in eukaryotic chassis cells. Compartmentalizing reaction segments that easily produce by-products will help improve efficiency of specific steroid transformations, and combined with P450 monooxygenase mutagenesis design, will produce synergistic effects for solving specificity problems.

(3) High Product Concentration Inhibition: Currently, P450 enzyme catalytic efficiency for steroid transformation is generally not high, so product inhibition at high concentrations has not been prominent. However, negative feedback inhibition of key catalytic proteins by products is a common phenomenon in product synthesis. As research progresses and steroid compound transformation efficiency continues to improve, high-concentration product inhibition will inevitably become an important production obstacle. This can be addressed by drawing on two-phase fermentation methods to transfer products timely to the organic phase, isolating them from the reaction system, or by enhancing endogenous efflux systems or introducing heterologous transport systems to promptly export products and avoid excessive intracellular product concentration. Additionally, using directed evolution and rational design to improve host or key enzyme tolerance to high product concentrations will also be an effective strategy.

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