Effects of Fsp27 Knockdown Combined with Myricetin Treatment on Lipolysis in 3T3-L1 Cells (Post-Print)

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Date: 2018-09-21T00:00:00+00:00

Abstract

Objective: To investigate the effects of downregulating fat-specific protein 27 (Fsp27) gene expression combined with myricetin intervention on lipid metabolism in 3T3-L1 cells, and to explore the regulatory mechanisms underlying the initiation and progression of lipid droplets.

Methods: 3T3-L1 preadipocytes were routinely cultured and induced to differentiate into mature adipocytes using the "cocktail" method. The sh-Fsp27 interference vector was transfected via the liposome method, and mature adipocytes were treated with complete medium containing 100 mol/L myricetin for 72 h. Oil Red O staining was performed to observe changes in lipid droplet morphology and size; enzymatic methods were used to determine intracellular glycerol and triglyceride contents to observe changes in cellular lipid metabolism. Western blot was used to detect the expression of Fsp27, hormone-sensitive triglyceride lipase (HSL), triglyceride lipase (ATGL), and mitogen-activated protein kinase (MAPK) signaling pathway proteins.

Results: 1. After induction and differentiation, 3T3-L1 cells changed from a fibroblast-like morphology to a round shape, accompanied by increased cell volume. 2. Compared with the control group, triglyceride content decreased and glycerol content increased in both the myricetin group and transfection group ($P<0.05$). Compared with the other three groups, the combined intervention group showed reduced triglyceride content and increased glycerol content ($P<0.05$). 3. Compared with the control group, Fsp27 protein expression decreased while ATGL and PPARγ expression increased in the other three groups ($P<0.05$). Additionally, HSL expression and the p-p38MAPK/p38MAPK ratio in both the combined intervention group and myricetin group were higher than those in the sh-Fsp27 group and control group ($P<0.05$).

Conclusion: 1. Combined intervention of Fsp27 gene silencing and myricetin

can promote lipolysis to a greater extent. 2. Myricetin exerts its lipolytic effect by activating the MAPK signaling pathway and upregulating HSL and ATGL protein expression; the sh-Fsp27 interference vector accelerates lipolysis by regulating PPARγ and Fsp27 protein expression and increasing ATGL content.

Full Text

Preamble

Engineering P450 for Specific Oxidation of Steroids

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Abstract

Steroidal drugs represent the second largest class of pharmaceuticals worldwide after antibiotics, possessing critical therapeutic activities such as anti-inflammatory, anti-allergic, and endocrine regulation. Effective and specific oxidation of the steroid nucleus at particular positions is essential for introducing pharmacological activity. Research has demonstrated that cytochrome P450 enzymes constitute a key 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 developments in the design and optimization of steroid-metabolizing P450 enzymes to guide in-depth research in this field.

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

Introduction

Steroidal drugs are the second largest class of pharmaceuticals globally, with over 400 varieties currently in production worldwide and global sales reaching $100 billion by 2017. Due to their significant regulatory functions in immune response, sexual function, glucose metabolism, and lipid metabolism, steroids are widely used to treat various inflammatory conditions, allergic reactions, and diseases affecting the respiratory, cardiovascular, endocrine, and oncological 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 steroid drug synthesis, specific oxidation of the steroid nucleus is a critical catalytic step for introducing pharmacological activity. This step commonly 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: see original paper]. Hydroxylation modification can increase drug polarity, thereby improving steroid solubility and plasma concentration, while also activating the substrate to facilitate subsequent catalytic reactions such as glycosylation. Consequently, hydroxylation is crucial for steroid drug activity.

Currently, steroid oxidation is 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. Therefore, an increasing number of researchers aim to leverage the efficiency and product specificity advantages of biocatalysis to achieve steroid nucleus oxidation, studying and optimizing endogenous steroid transformation systems and gradually introducing key enzyme systems into heterologous model organisms to construct efficient steroid transformation platforms.

Research has shown that the vast majority of steroid modifications in organisms are mediated by cytochrome P450 monooxygenases, which represent the most important enzyme system for steroid oxidation reactions. These enzymes utilize the cofactor NADP(H) as an electron donor and leverage the oxidizing capability of heme iron to achieve targeted monooxygenation reactions. However, researchers often cannot achieve ideal steroid oxidation effects through simple expression of native P450 monooxygenases.

This limitation arises 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 native 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 NADP(H) to the catalytic center' s ferrous heme to complete steroid substrate oxidation. The interaction between these proteins and endogenous or heterologously expressed electron transfer systems 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 steroid modification efficiency and product specificity. Current strategies primarily address P450 monooxygenase expression issues [FIGURE:2, white section] through: (1) modifying the P450 enzyme N-terminus to improve effective localization and soluble expression in heterologous hosts; (2) assisting correct folding of P450 monooxygenases through chaperone co-expression to ensure catalytic activity; and (3) introducing site-directed mutations to enhance P450 monooxygenase expression levels and catalytic efficiency. 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 research efforts to enhance catalytic reaction efficiency by strengthening electron transfer efficiency and improve catalytic specificity by optimizing key P450 monooxygenase structures [FIGURE:2, orange section]. We describe in detail how engineered heterologous P450 catalytic systems can achieve efficient and specific oxidation of the steroid nucleus in model microbial hosts, providing guidance for related 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 3A: see original paper].

The Class I P450 electron transfer system consists of ferredoxin (Fdx) containing iron-sulfur clusters paired with ferredoxin reductase (FdR) containing FAD cofactors, or alternatively flavodoxin (Fld) paired with flavodoxin reductase (FpR). The most widely applied system is adrenodoxin (Adx) and adrenodoxin reductase (AdR) from mammalian mitochondria. The Adx/AdR electron transfer system can support not only mitochondrial P450 enzymes from various eukaryotes 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-P450 reductase (CPR) with FAD and FMN prosthetic groups [Figure 3A: see original paper]. 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 cytoplasm. Electron transfer occurs through random molecular collisions between the two components.

During heterologous expression of P450 catalytic systems, low heterologous expression levels, poor activity, or difficult synergistic interactions between oxidases and reductases often result in low electron transfer efficiency, greatly

inhibiting P450 oxidase catalytic efficiency. To address these issues, researchers typically employ three strategies [Figure 3: see original paper]: (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 these strategies in the context of practical problems encountered when constructing heterologous P450 catalytic systems for steroid nucleus oxidation.

1.1 Matching P450 Oxidases with Redox Partners

Co-expressing P450 oxidases with redox partners that exhibit high coupling efficiency is an effective means to improve electron transfer efficiency in P450 catalytic systems. To achieve this goal, researchers often screen combinations of redox partners from different sources with target P450 oxidases [Figure 3B: see original paper]. Screening typically begins by 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*), among which Etp1fd/Arh1 is highly homologous to Adx/AdR.

Based on successful cases of improved steroid ring oxidation efficiency listed in , we find that redox partners exhibiting high coupling efficiency with 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*, humans, and endogenous fission yeast reductases, human CYP17 and CYP21 showed highest catalytic activity when matched with endogenous fission yeast CPR. This may be because isozymes from different species inherently exhibit 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 approach 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 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 the Class II CPR 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, achieving ER localization. Under the action of endogenous yeast CPR, electrons were successfully transferred to CYP27 to accomplish 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 endogenous environment of the host 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 partners. Further mechanistic analysis of these examples will aid in designing and constructing heterologous P450 catalytic systems for efficient steroid oxidation.

1.2 Supplementing Redox Power

As previously mentioned, 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 , two primary 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: see original paper]. These strategies enhance electron transfer efficiency by regulating electron flow.

1.2.1 Adjusting the Ratio of Oxidase to Redox Partner Regulating the expression ratio of protein subunits in the P450 enzyme system is a common method for controlling redox power [Figure 3C: see original paper]. For example, Sawada et al. expressed CYP105A1 from *Streptomyces griseolus* with spinach ferredoxin and its reductase Fdx/FdR in *E. coli* to convert VD2 to 1 ,25-(OH)VD2. Their results showed that co-expression at a ratio of 4 µM Fdx/0.2 µM FdR yielded significantly higher CYP105A1 catalytic activity than a 4 µM Fdx/2 µM FdR ratio. 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%. These results demonstrate that for Class I P450 redox systems, maintaining high electron transfer efficiency requires a high ferredoxin/ferredoxin reductase ratio, likely because ferredoxin plays a bridging role in Class I P450 electron transfer chains.

1.2.2 Expressing Additional Electron Transfer Components Expressing additional electron transfer components is a common method for supple-

menting redox power, particularly when constructing Class II P450 catalytic systems. Researchers often express additional cytochrome b5 (Cyt-b5) [Figure 3C: see original paper]. 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 at 10 times the rate of CPR, and electron transfer chain coupling increases 5-fold upon Cyt-b5 introduction. However, Sang et al. reported that Cyt-b5 introduction can alter the conformation of the CYP2B4 catalytic center when catalyzing methoxyflurane, with the degree of change depending on the CPR/Cyt-b5 ratio. Therefore, Cyt-b5 application in steroid oxidation reactions requires careful consideration, with expression ratios adjusted as necessary.

1.3 Artificial Assembly of P450 Oxidases and Redox Partners

Both eukaryotic and prokaryotic organisms contain naturally occurring P450 oxidase-CPR fusion proteins that utilize fused electron transfer chains for more efficient electron transfer, with rates exceeding those of independently expressed P450 isozymes by more than two orders of magnitude. The archetypal example is P450 BM3 (CYP102A1), whose fatty acid hydroxylation reaction rate is significantly higher than eukaryotic fatty acid hydroxylases. Therefore, fusing P450 oxidases with their redox partners represents a strategy to enhance electron transfer efficiency by shortening spatial distance [Figure 3D: see original paper].

For instance, the reductase component of the natural fusion protein P450 BM3 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 from *Bacillus megaterium* using a linker. The resulting fusion enzyme showed 6% higher catalytic activity toward dextromethorphan than non-native redox partner-reconstituted CYP130. For Class I P450 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 reductase (PdR), using free putidaredoxin (PdX) as the electron transfer component, which increased camphor 5-hydroxylation efficiency to twice that of the natural system. Thus, fusion expression of P450 oxidases with their redox partners helps improve electron transfer levels and catalytic efficiency.

Despite the effectiveness of fusion expression, recent attempts to fuse steroid oxidases with their redox partners have struggled to improve catalytic efficiency. Charles et al. fused bovine CYP17A with rat P450 reductase to catalyze 17-hydroxylation of pregnenolone, but the fusion protein showed no 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 using three different linkers, finding that all fusion proteins had kcat values far lower than separately expressed CYP11A1. Structural analysis of the Adx/CYP11A1 complex revealed that electron transfer depends on specific orientation-dependent interface interactions. Fusion of Adx with AdR may hinder these specific interface interactions, preventing effective electron transfer between the Adx iron-sulfur cluster and CYP11A1 heme.

These results suggest that for Class I P450 systems containing ferredoxin, protein fusion strategies should consider interface interaction-based electron transfer processes, and analysis of subunit interactions can 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. The resulting non-covalent heterotrimeric complex showed 50-fold higher NADPH and O2 consumption rates than independently expressed P450cam, PdX, and PdR. Therefore, developing assembly strategies for P450 oxidases and their redox partners may effectively address the current challenge that fusion expression rarely produces positive effects in steroid P450 catalytic systems.

2. Improving Catalytic Specificity of Engineered Steroid P450 Monooxygenases

Due to the high conservation of the steroid nucleus 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 simultaneously possesses hydroxylation activity at steroid ring positions C6, C9, and C15, while CYP27A1 can hydroxylate positions C25, C26, and C27. Practical applications require specific oxidation at particular steroid ring positions to exploit the unique functional activity of catalytic products. To meet these requirements, target P450 proteins must be engineered and optimized to obtain steroid P450 monooxygenases with excellent catalytic specificity. Current strategies for P450 modification and optimization are divided into semi-rational and rational design, with irrational design being less commonly applied. We discuss these approaches separately below.

2.1 Directed Evolution of Steroid P450 Monooxygenases Based on Irrational Design

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 a 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 efficiency remains low. This is because irrational design typically focuses on improving expression levels and catalytic efficiency rather than specificity. Additionally, 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, leading researchers to employ semi-rational or rational design approaches.

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

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 foundation for P450 research and modification. Semi-rational design analyzes these complex structures to predict several residues affecting P450 monooxygenase catalytic selectivity, followed by saturation mutagenesis to enhance specific oxidation at target steroid ring positions.

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 in vitro conversion experiments. 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, initially identifying residues S225, S275, and S276 as directly related to CYP260A1 catalytic selectivity toward progesterone. Saturation mutagenesis at these 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 Directed Evolution of Steroid P450 Monooxygenases Based on Rational Design

Rational design is based on comprehensive understanding of steroid P450 monooxygenase protein structures and catalytic mechanisms, enabling precise regulation of target protein structures to alter catalytic selectivity. In rational design, mutation site selection involves analyzing P450 protein-steroid substrate complex structures and comparing homologous sequences between isozymes with different catalytic selectivities to identify potential specificity-determining residues.

For example, when studying substrate selectivity for CYP17A1-catalyzed C17-C20 bond cleavage, Gregory et al. compared homologous sequences of CYP17A1 from eight different vertebrates, finding that when residue 202 was asparagine, the enzyme favored substrates with hydroxyl groups at steroid ring C3 (e.g., 17-hydroxypregnenolone), whereas serine or threonine at position 202 favored substrates with keto groups at C3 (e.g., 17-hydroxyprogesterone). They therefore proposed that residue 202 determines CYP17A1 substrate selectivity, with the human CYP17A1 N202S mutant reversing 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 weaker C16 hydroxylases 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. Thus, comparing homologous sequences of isozymes with different catalytic specificities and mutating conserved residues from one specificity class to another represents a simple and effective strategy for altering steroid P450 catalytic selectivity without requiring protein complex structural information.

As shown in , key amino acid residues determining steroid P450 catalytic selectivity are mostly located at or near the catalytic active center. Mutations at these residues 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. Based on cases listed in , strategies for altering catalytic selectivity can be divided into three categories [Figure 4: see original paper]:

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

As shown in , successful cases of altering steroid P450 monooxygenase catalytic specificity over the past decade have primarily involved the A, C, and D rings of the steroid nucleus, with fewer cases involving the B ring. This may be due to greater spatial hindrance between the B ring and the heme. This suggests that

changing the length of amino acid side chains at specific positions can adjust spatial hindrance to alter the relative position between the substrate catalytic site and the P450 active center heme, 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 C16 and reducing byproduct 16-hydroxyprogesterone formation. Paolo et al. sequentially mutated amino acid residues with small nonpolar side chains in the CYP3A4 active center to larger nonpolar residues (tryptophan or phenine) based on their distance from the heme, reducing progesterone binding capacity. While wild-type CYP3A4 hydroxylates progesterone to produce large amounts of 6 -hydroxyprogesterone and small amounts of 2 -hydroxyprogesterone plus other byproducts, mutant I301F/I369F/L482F balanced C2 and C6 hydroxylation capacity and halved byproduct proportions. The large side chain' s rigid topology structure reduces ligand binding freedom, thereby restricting specific catalytic sites.

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

Mutating key residues to amino acids 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, when hydroxylating 17-hydroxyprogesterone at C11 in *E. coli*, Xiong et al. mutated human CYP11B1 L382 to serine (containing a hydroxyl side chain). The L382S mutation formed a new hydrogen bond with the heme carboxyl group, shifting the heme toward the substrate' s C11 position. Combined with the flexibility of the adjacent F381A mutation, this enhanced the interaction between L382S and the heme [Figure 4B: see original paper]. 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 enhance target oxidation specificity while simultaneously improving steroid P450 monooxygenase catalytic efficiency.

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

Mutating amino acid residues involved in substrate binding can alter steroid substrate spatial orientation in the P450 active center by enhancing or weakening hydrogen bonding or hydrophobic interactions, thereby adjusting the distance between target sites and the heme to regulate catalytic selectivity. For example, Khatri et al. found that progesterone can bind to the CYP260A1 catalytic active center in two different spatial orientations [Figure 4C: see original paper]. When progesterone' s C3- and C20-keto groups form hydrogen bonds with S225 and S276 of N-terminally truncated CYP260A1 (tCYP260A1), the substrate C1 position is near the heme and CYP260A1 favors C1 catalysis (spatial position 1). When the hydrogen bonds involve S276 and S225 instead, the substrate'

s relative spatial position in the active center is reversed, with C17 near the heme and CYP260A1 favors C17 catalysis (spatial position 2). The CYP260A1 S276N mutant disrupted the hydrogen bond between wild-type S276 and progesterone' s C3-keto group, preventing stable binding in spatial position 2 and thus producing primarily 1-hydroxyprogesterone. Conversely, mutant S276I did not affect spatial position 2 binding (the I276 side chain could form hydrophobic interactions with the A ring to compensate for lost hydrogen bonding), but its larger hydrophobic side chain hindered hydrogen bond formation with the C20-keto group, preventing stable binding in spatial position 1 and thus producing primarily 17-hydroxyprogesterone.

Conclusion and Perspectives

Steroid biotransformation research has a history of nearly 70 years, providing mature understanding of steroid biosynthetic pathways and key enzymes, particularly cytochrome P450 monooxygenases. With scientific and technological advances, engineering P450 monooxygenases can effectively improve steroid oxidation modification efficiency and selectivity. Current research reveals 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 systems in electron transfer while facilitating regulation of electron supply balance. New electron supply systems can also 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 compound oxidation modification.

Additionally, 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 established research approaches:

(1) Low Aqueous 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 concentrations. However, cosolvent addition may damage host cell membranes and cause P450 protein denaturation. For membrane damage, expression of relevant genes (e.g., lipid metabolism genes) can be regulated to improve membrane stability. For enzyme denaturation, protein mutagenesis and recombination can obtain solvent-tolerant mutants. Furthermore, with the realization of de novo synthesis of steroid drug intermediates such as progesterone and hydrocortisone, synthetic biology is promoting a transition from biotransformation to "synthesis from simple carbon sources using artificially engineered functional microorganisms," which can also avoid the low substrate availability issue.

(2) Poor Modification Specificity: Constructing heterologous steroid expression pathways involves multi-step consecutive P450-catalyzed reactions. In practice, non-specific substrate catalysis creates complex network catalytic pathways, making P450 catalytic selectivity issues more prominent and posing enormous challenges for P450 mutagenesis design. While we have systematically described using rational, semi-rational, and irrational design to mutate amino acid residues and solve specificity issues, it is difficult to rely solely on directed evolution of individual P450 proteins to address network synthesis pathways. However, research shows that enzymes in natural eukaryotic sterol or steroid synthesis pathways are often localized to different organelles, providing excellent justification for compartmentalized reaction design in eukaryotic chassis cells. Compartmentalizing reaction steps prone to byproduct formation can help improve efficiency of specific steroid transformations, and combined with P450 monooxygenase mutagenesis design, can produce synergistic effects.

(3) High Product Concentration Inhibition: Currently, P450 enzyme-catalyzed steroid transformation efficiency is generally not high enough for product inhibition to be prominent. However, as research advances and transformation efficiency improves, high product concentration inhibition will inevitably become a significant production bottleneck. This can be addressed by adopting two-phase fermentation methods to transfer products to the organic phase in real-time, 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 accumulation. Additionally, directed evolution and rational design methods to improve host or key enzyme tolerance to high product concentrations will also be effective strategies.

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