Engineering Cytochrome P450 biocatalysts for biotechnological applications

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• Introduction
• Biotechnological interesting aspects
• Limitations and engineering targets
• Engineering strategies
• A Novel P450 with biotechnological properties
• Conclusion
Cytochrome P450, external monooxygenases

RH + O₂ + H⁺ + NAD(P)H \text{Cytochrome P450} \rightarrow ROH + NAD(P)⁺ + H₂O

Soret peak at 450 nm in their reduced form when saturated with carbon monoxide.
Cytochrome P450, external monooxygenases

\[ \text{RH} + \text{O}_2 + \text{H}^+ + \text{NAD(P)H} \xrightarrow{\text{Cytochrome P450}} \text{ROH} + \text{NAD(P)^+} + \text{H}_2\text{O} \]
Structural aspects

External monooxygenases: need for external electron donor
Various classes with respect to the nature of the reductase domain

Introduction
Introduction

(1) Hydrocarbon hydroxylation

(2) Alkene epoxidation/Alkyne oxygenation

(a) \[ \text{\ring{C}=\text{C}} \rightarrow \text{\ring{C}\text{-OH}} \]

(b) \[ \text{\ring{H-C\text{-C}}=\text{O}} \rightarrow \text{\ring{R\text{-C}}\text{-CO}} \]

(3) Arene epoxidation, aromatic hydroxylation, NIH shift

(4) N-Dealkylation

(5) S-Dealkylation

(6) O-Dealkylation

(7) N-Hydroxylation

(8) N-Oxidation

(9) S-Oxidation

(10) Oxidative deamination

(11) Oxidative dehalogenation

(12) Alcohol and Aldehyde oxidations

(a) \[ \text{\ring{H-C\text{-OH}}} \rightarrow \text{\ring{R\text{-C}}\text{-CO}} + \text{R(H)} \]

(b) \[ \text{\ring{R\text{-C}}\text{-CO}} \rightarrow \text{\ring{R\text{-C}}\text{-CO}} + \text{H}_{2}\text{O} \]
Introduction

(13) Dehydrogenation

\[
\begin{align*}
\text{(a)} & & \text{CH}_2\text{CH}_2 \rightarrow & & \text{CH}_2\text{CH}^- \rightarrow & & \text{CH}_2\text{CH}^- \\
\text{(b)} & & \text{NH Ac} & & \text{HN Ac} \\
\text{HO} & & \text{HO} & & \text{CO}
\end{align*}
\]

(14) Dehydrations

\[
\begin{align*}
\text{(a)} & & \text{H}_2\text{C} = \text{N} - \text{OH} \rightarrow & & \text{R} - \text{C} = \text{N} & & \text{H}_2\text{O} \\
\text{(b)} & & \text{R} - \text{C} = \text{O} \rightarrow & & \text{R} - \text{C} = \text{C} - \text{O} & & \text{H}_2\text{O}
\end{align*}
\]

(15) Reductive dehalogenation

\[
\begin{align*}
\text{R} - \text{C} - \text{X} & + \text{e}^- \rightarrow & \text{R} - \text{C} - \text{R} & + \text{X}^- \\
\end{align*}
\]

(16) N-Oxide reduction

\[
\begin{align*}
\text{N} = \text{O}^+ & + 2\text{e}^-; (+2\text{H}_2^+) \rightarrow \text{N} (+\text{H}_2\text{O})
\end{align*}
\]

(17) Epoxide reduction

\[
\begin{align*}
\text{R} & + 2\text{H}_2^+; +2\text{e}^- \rightarrow \text{CH}_2\text{CH}_2 & + \text{H}_2\text{O}
\end{align*}
\]

(18) Reductive β-scission of alkyl peroxides

\[
\begin{align*}
\text{X} - \text{C} - \text{C} - \text{O} - \text{OH} & + 2\text{H}_2^+, +2\text{e}^- \rightarrow \text{X} - \text{O} + \text{R} - \text{H} + \text{H}_2\text{O}
\end{align*}
\]

(19) NO reduction

\[
\begin{align*}
\text{O} - \text{N} = \text{N} & - \text{O} + 2\text{e}^-; +2\text{H}_2^+ \rightarrow \text{N} = \text{N} - \text{O} + \text{H}_2\text{O}
\end{align*}
\]

(20) Isomerizations

Prostaglandin H₂ (PGH₂)

Thromboxane A₂ (TXA₂)

(21) Oxidative C-C bond cleavage

\[
\begin{align*}
(1) & & \text{R} & + \text{H}_2\text{O} & + \text{O}_2 & \rightarrow \text{R} - \text{H} + & \text{H}_2\text{O} \\
(2) & & \text{R} & + \text{H}_2\text{O} & + \text{O}_2 & \rightarrow \text{R} - \text{H}_2\text{O} + \text{H}_2\text{O}
\end{align*}
\]
Cytochrome P450, a versatile biocatalyst

Therapy of diseases (e.g. fungal infection)

Drug Developments, Metabolite Production, Biotransformation of drugs

Fine chemical Synthesis, Stereo- and regioselective hydroxylation

Medicine

Pharmacology

Chemistry

Toxicology

Plant sciences

Color, Flavor, Secondary metabolite production

Microbiology

Biotransformation of xenobiotics

Environmental Sciences

Bioremediation

Degradation of terpenes, alkanes, fatty acids

Environmental Sciences
Biotechnological interesting aspects

✓ Catalytic versatility
✓ Substrate diversity
✓ Sheer number, over 21,000 different CYPs found in all kingdoms
✓ High regio- and stereo selectivity on complex molecules, hard to obtain with chemical synthesis
✓ Oxidation of inert C-atoms under normal pressure and at room temperature
**Limitations and engineering targets**

- Low activity, most cases $k_{\text{cat}}$ values in the range of $\sim 1–300 \text{ min}^{-1}$
- Substrate specificity
- Cofactor requirements, e.g. NAD(P)H
- Ineffective electron flow, uncoupling
- Organic solvent intolerance
- Poor stability during isolation, storage and use

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**Diagram:**

- **Heme domain**
- **Reductase domain**

**Notes:**

- Stability
- Activity
- Solvent tolerance
- Electron transfer/uncoupling
- Specificity
Engineering strategies

- Molecular modeling
- Site directed mutagenesis

- Semi-rational Design
- De novo Design
- Conserved Sequence Motif Analysis (CSM)
- Chimeragenesis
- ...

Combined with HTS

E.g. epPCR
Novel P450 with biotechnological properties

Single-step fermentative production of the cholesterol-lowering drug pravastatin via reprogramming of *Penicillium chrysogenum*

Kirsty J. McLean et. al. (2015)

- decrease the risk of cardiovascular disease (CVD) and brain strokes
- Inhibition of 3β-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase → reduce “bad” plasma (LDL) cholesterol levels
- effective against hypercholesterolemia
Novel P450 with biotechnological properties

Single-step fermentative production of the cholesterol-lowering drug pravastatin via reprogramming of

*Penicillium chrysogenum*

Kirsty J. McLean et. al. (2015)

A

**Compactin**
Aspergillus terreus and
Penicillium citrinum

**Pravastatin**

Stereoselective hydroxylation
Industrial pravastatin production

- costly dual-step fermentation and biotransformation process,
- low P450sca-2 activity during biotransformation,
- compactin toxicity,
- and low pravastatin yield.
Industrial pravastatin production

One-step fully fermentative production process.

Reprogrammed *Penicillium chrysogenum* expressing engineered CYP105AS1 of *Amycolatopsis orientalis*
Choice & preparation of test system

β-lactam antibiotics producer *Penicillium chrysogenum*

- numerous rounds of classical strain improvement led to current penicillin production titers of more than 50 g/L
- industrial robustness, high flux from glucose to secondary metabolites
- But undesirable penicillin producing gene clusters
  - Deletion of all penicillin biosynthetic genes required
  - Further transformation with compactin (ML-236B) gene cluster from *P. citrinum*
Several strains produced up to 624 mg/L statin vs. natural compactin producer *p. citrinum* with 19 mg/L.

- high ratio of deacylated (ML-236A) vs. acylated (compactin) statins

imbalance in the introduced pathway or a competing activity?
Preparation of producing system

- Several strains produced up to 624 mg/L statin vs. natural compactin producer *p. citrinum* with 19 mg/L.
- High ratio of deacylated (ML-236A) vs. acylated (compactin) statins
  - Imbalance in the introduced pathway or a competing activity?
- A putative esterase encoded by *Pc15g00720* gene in statin deacylating transformants
Identification of an effective hydroxylase

No pravastatin production when using P450sca in \textit{p.chrysogenum}

➤ finding a new hydroxylating enzyme with a higher hydroxylation activity on compactin
- No available system with 100% conversion rate or better conversion over \textit{S.carbophilus}

✓ up to 100% hydroxylation activity in \textit{Amycolatopsis orientalis}
   ➢ Confirmation of a CYP enzyme as a compactin hydroxylase, the CYP105AS1

❗ But exhibiting low activity when expressed in \textit{p.chrysogenum}
Expression of CY105AS1 in *P. chrysogenum*

Functionality of CYP105AS1 in *P. chrysogenum* is desired

Optimization of electron flow:

- Effective interaction between redox partner and heme domain crucial

→ Use of reductase domains of self-sufficient CYPs, such as RhF from *Rhodococcus* sp.
A self-sufficient fusion enzyme

- Fusion of a codon-optimized CYP105AS1 to RhF reductase domain,
- Expression of the hybrid gene under the strong pcbC promoter

- Up to 550mg/L pravastatin, including 98-100% 6-epi-pravastatin!!
uninterpretable electron density in the active site

- ample space for the substrate to occupy multiple configurations.

→ Low stereo-selectivity of WT

⚠ Change in protein structure to limit the landscape of the substrate!

✓ Mutation by error-prone PCR
✓ Screening for higher Pravastatin producing mutants
Evolution of CYP105AS1

<table>
<thead>
<tr>
<th>Ao CYP mutant</th>
<th>First round: Error-prone PCR</th>
<th>Second round: Site saturation combined with error-prone PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CYP105AS1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>I95T</td>
<td>F24L, A180L, H132R, I95A, I95T</td>
</tr>
<tr>
<td>#2</td>
<td>I95T</td>
<td></td>
</tr>
<tr>
<td>#3</td>
<td>I233T A180T</td>
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<tr>
<td>#4</td>
<td>L236P A388T</td>
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<tr>
<td>#5</td>
<td>A180V</td>
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</tr>
<tr>
<td>#6</td>
<td>L236I A265V</td>
<td></td>
</tr>
<tr>
<td>#7</td>
<td>A180L A265C</td>
<td></td>
</tr>
</tbody>
</table>

Single amino acid change altered stereoselectivity

- Engineering a 6-**epi**-pravastatin producing CYP450 into a pravastatin synthase (P450<sub>Prava</sub>), with only two rounds of mutagenesis and three to five amino acid changes

mutants with corresponding pravastatin: 6-**epi**-pravastatin production ratios up to 96:4
The WT CYP105AS1 open active site

Pravastatin synthase including I95T, L236I, A180V near active site and Q127R, A265N mutations far from the active site.
Expression of $P450_{prava}$ in a compactin production strain

- Fusion of $P450_{prava}$ to Rhf reductase and transformation in P. chrysogenum compactin strain
- Proof in Small scale
  - Transformants with 688 mg/L

Proof in larger scale
- 10L fed-batch fermentation
- After 200h, >6g/L pravastatin production vs. Classical two-step production with 2-3 g/L pravastatin production.
Conclusion

Great interest for use of Cytochrome P450 in industry

• versatility for a wide substrate range
• performing large number oxidative reactions
• specifically addition of oxygen atoms at challenging positions on chemical scaffolds

→ Identification and overcoming the limitations crucial
e.g. NAD(P)H regeneration by applying metabolomics and fluxomics techniques

New perspective in producing important molecules through microbial fermentation processes
Thank you for your attention
References