Recent progress on the application of cytochrome P450 (P450) to bioconversion processes, biosensors, and bioremediation were reviewed. Because regio- and enantioselective hydroxylation makes chemical synthesis difficult, a bioconversion process using P450 would be quite attractive. One of the most successful industrial applications of P450 may be the bioconversion process for pravastatin formation using a Streptomyces carbophilus CYP105A3. Unfortunately, practical application of P450s in the bioconversion process is limited because of their low stability, low activity and co-factor dependency. However, directed evolution is expected to generate useful P450 biocatalysts for a wide range of substrates. Shunt pathways of CYP152A1, CYP152A2, and CYP152B1 are notably promising for practical application, because these P450s require neither reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) nor electron donor proteins, and efficiently catalyze using hydrogen peroxide. A P450 biosensor using biochip technology is expected to become a tool for rapidly determining drugs and endogenous substances in plasma at a low cost. Bioremediation of dioxins and polychlorinated biphenyls (PCBs) by the CYP1 family appears to be possible by using suicidal, genetically engineered microorganisms. The P450 superfamily has tremendous potential for practical applications in various fields.

Keywords: P450; practical application; bioconversion; biosensor; bioremediation

INTRODUCTION

Cytochrome P450 (P450) catalyzes various types of reactions such as hydroxylation, epoxidation, alcohol and aldehyde oxidation, O-dealkylation, N-dealkylation, oxidative dehalogenation, and oxidative C–C bond cleavage. Among these, regio- and enantioselective hydroxylation by P450 is quite attractive as a bioconversion process to produce drugs, vitamins, flavors, fragrances, and pesticides. The introduction of one hydroxyl group often dramatically changes the properties of the compound, such as its solubility, efficacy, and toxicity. One of the most successful examples is the bioconversion of compactin to pravastatin by CYP105A3-dependent 6β-hydroxylation. However, most P450 reactions are slow, with $k_{\text{cat}}$ values of less than $100 \text{ min}^{-1}$, although CYP102A1-dependent ω-2 hydroxylation of myristic acid is more than 4,000 min$^{-1}$. Figure 1 shows the reaction cycle of P450. Usually, the rate-limiting step of the P450 reaction cycle is considered to be electron transfer from electron donor proteins. The extraction of a hydrogen atom from the substrate or release of the product could also be the rate-limiting step (Fig. 1). It is noted that CYP102A1, a fusion protein containing both P450 and reductase domains in one polypeptide chain (Fig. 2), shows remarkable catalytic activity. However, the application of P450s for the production of bulk chemicals is quite difficult because of their low stability, low activity, and co-factor dependency. This is the disadvantage of oxidoreductases as biocatalysts, compared with hydrolases that are the most widely used enzymes. Based on these aspects, practical application of P450 in the bioconversion process may be limited to the production of fine chemicals such as pharmaceuticals, flavors, and fragrances.

This paper reviews the recent advancements and future prospects of the practical use of P450s as biocatalysts.

1. APPLICATION OF P450 TO THE BIOCONVERSION PROCESS

One of the most successful applications of P450 on an
industrial scale appears to be the bioconversion process for pravastatin production, developed by Sankyo (present Daiichi-Sankyo) Co., Ltd. Pravastatin is a potent low-density lipoprotein (LDL)-cholesterol-lowering drug prescribed to several millions of hyperlipidemic patients in more than 100 countries. The lead compound ML-236B (compactin) found in Penicillium citrinum is converted to pravastatin through 6β-hydroxylation with fermentation by Streptomyces carboxiphilus. Watanabe et al. reported that this process was catalyzed by a water-soluble P450 named P450sca-2 (CYP105A3) (Table 1).

Another successful industrial application of P450 is the actinomycete P450-dependent conversion of vitamin D3 to 1α,25-dihydroxyvitamin D3 which is an important medicine for hypothyroidism, osteoporosis, and chronic renal failure. Since the chemical synthesis of 1α,25(OH)2D3 requires approximately 20 steps with a low yield, simple bioconversion for 1α,25(OH)2D3 production is quite attractive. Sasaki et al. developed the transformation of 25- and 1α-hydroxyvitamin D3 to 1α,25(OH)2D3 by Streptomyces sp., and the transformation of vitamin D3 to 1α,25(OH)2D3 via 25(OH)D3 by Amycolata sp. The gene encoding for vitamin D3 25-hydroxylase was cloned from Amycolata autotrophica, which has been renamed Pseudonocardia autotrophica.

Directed evolution study of CYP107 has significantly enhanced its activity. We also found that Streptomyces griseolus CYP105A1 can convert vitamin D3 to 1α,25(OH)2D3. Site-directed mutagenesis of CYP105A1 based on its three-dimensional structure dramatically enhanced the activity. The mutant exhibited approximately 400- and 100-fold higher kcat/Km values for the 25-hydroxylation and 1α-hydroxylation of vitamin D3, respectively, than the wild type enzyme.

Steroids are widely used as important medicines. Methods for the industrial production of steroids have been developed by combining chemical synthesis and microbial conversion steps. Microbial steroid bioconversions are established.

Table 1. Practical Application of P450s to Bioconversion Processes

<table>
<thead>
<tr>
<th>CYP</th>
<th>Species</th>
<th>Substrate</th>
<th>Reaction</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>105A3</td>
<td>S. carboxiphilus</td>
<td>Compactin</td>
<td>6β-Hydroxylation</td>
<td>3</td>
</tr>
<tr>
<td>107a</td>
<td>P. autotrophica</td>
<td>Vitamin D3</td>
<td>25- and 1α-Hydroxylation</td>
<td>7</td>
</tr>
<tr>
<td>105A1b</td>
<td>S. griseolus</td>
<td>Vitamin D3</td>
<td>25- and 1α-Hydroxylation</td>
<td>10</td>
</tr>
<tr>
<td>106A2</td>
<td>B. megaterium</td>
<td>Progesterone</td>
<td>11α-Hydroxylation</td>
<td>19</td>
</tr>
<tr>
<td>102A1</td>
<td>B. megaterium</td>
<td>Alkanes</td>
<td>Hydroxylation</td>
<td>27, 28</td>
</tr>
<tr>
<td>109B1</td>
<td>B. subtilis</td>
<td>(+)Valencene</td>
<td>C2-Oxidation</td>
<td>20</td>
</tr>
<tr>
<td>152A1, A2, B1</td>
<td>B. subtilis</td>
<td>Fatty acids</td>
<td>α- or β-Hydroxylation</td>
<td>32–34</td>
</tr>
<tr>
<td>152A1</td>
<td>B. subtilis</td>
<td>Styrene</td>
<td>Epoxidation</td>
<td>35</td>
</tr>
<tr>
<td>P450sca-2b</td>
<td>C. lunata</td>
<td>11-Deoxy cortisol</td>
<td>11β-Hydroxylation</td>
<td>12, 13</td>
</tr>
<tr>
<td>11B1</td>
<td>Bovine</td>
<td>11-Deoxy cortisol</td>
<td>11β-Hydroxylation</td>
<td>18</td>
</tr>
<tr>
<td>17A1, 21B2</td>
<td>Bovine</td>
<td>Progesterone</td>
<td>17α- and 21-Hydroxylation</td>
<td>14</td>
</tr>
<tr>
<td>11A1, 17A1</td>
<td>Bovine</td>
<td>Endogenous sterols</td>
<td>Sequential hydroxylation to cortisol</td>
<td>17</td>
</tr>
<tr>
<td>11B1, 21A2</td>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) This P450 belongs to CYP107 family, but CYP name was not given. b) Multiple amino acids were substituted. c) Short-alkyl-chains carboxylic acids were used as decoy molecules. d) Primary structure of P450sca-2 has not yet been reported.
methods in the steroid industry.\textsuperscript{11} For example, immobilized Curvularia lunata mycelium that was applied by Schering AG (Germany) for producing cortisol from 11-deoxycortisol was due to P450lun-dependent 11β-hydroxylation.\textsuperscript{12,13} The efficacy of steroid bioconversion by a microorganism might be improved by the overexpression of the P450 responsible for the target reaction. Steroidogenesis from cholesterol to cortisol in the adrenal cortex involves hydroxylation reactions catalyzed by CYP11A1, CYP17A1, CYP21A2 (or CYP21B2) and CYP11B1. We attempted the generation of recombinant yeast cells applicable to steroid bioconversion. Bovine CYP17A1 and CYP21B2 cDNAs were co-expressed in S. cerevisiae cells.\textsuperscript{14} These recombinant yeast cells could convert progesterone to 11-deoxycortisol by sequential 17α- and 21-hydroxylations. This result suggested the possibility of a practical application of such recombinant yeast cells. We also examined the construction of fused enzymes between CYP17A1 or CYP21B2 and yeast reduced nicotinamide adenine dinucleotide phosphate (NADPH)-P450 reductase to enhance the activity.\textsuperscript{15,16} Szczecbura et al.\textsuperscript{17} expressed bovine CYP11A1, bovine CYP17A1, human CYP21A2, human CYP11B1, bovine adrenodoxin (ADX), bovine adrenodoxin reductase (ADR), and bovine 3β-hydroxysteroid dehydrogenase cDNAs simultaneously in S. cerevisiae cells, and succeeded in producing cortisol from endogenous sterols of the yeast cells. Hakki et al.\textsuperscript{18} expressed bovine CYP11B1 in the fission yeast S. pombe, and successfully converted deoxycortisol to cortisol. These results suggest that the yeasts S. cerevisiae and S. pombe are useful hosts for constructing recombinant cells expressing mammalian P450s for producing steroid hormones for medical use.

Recently, the Bernhardt group has succeeded in the expression of Bacillus megaterium CYP106A2 and its reducing enzymes in Escherichia coli cells to produce hydroxylated steroid derivatives.\textsuperscript{19} Since the activity and substrate-specificity of this system are expected to be modified by site-directed mutagenesis of this P450, this bacterial system might have great potential to produce known or novel steroidal derivatives.

Oxygenated terpenes are important materials in the flavor and fragrance industries. Most enzymes catalyzing the regio- and enantioselective hydroxylation of terpenes are P450s. The regiospecific hydroxylation of terpenes by chemical reaction is difficult due to similar electronic properties of the primary and secondary allylic positions, and unexpected epoxidation. Recently, Girhard et al.\textsuperscript{20} successfully converted (+)-valencene to (+)-nootkatone by recombinant E. coli cells co-expressing B. subtilis CYP109B1 and its reducing enzymes. The productivity of (+)-nootkatone by this system\textsuperscript{20} was 15 mg L\textsuperscript{-1} h\textsuperscript{-1}, although its production from grapefruit peels was a costly process. Industrial applications of P450-dependent bioconversions have been made on the production of valuable fine chemicals. In order to apply P450 to produce more general chemicals with low prices, further efforts described below will be required.

(1) Screening and Protein Engineering of P450 Screening of suitable P450s from the genome or cDNA libraries and their expression in appropriate hosts are the first steps necessary to establish a bioconversion process using P450. The next step is the modification of selected P450 by protein engineering, such as site-directed mutagenesis and the construction of chimeric P450 to improve its catalytic properties. Directed evolution involving random mutagenesis and DNA shuffling is also a powerful method.\textsuperscript{21,22} Combination of rational design based on the structural information obtained by X-ray crystallographic analysis and directed evolution may be the best way to generate a highly active P450. Construction of an efficient electron transfer system including fusion proteins with P450 reductase is another way to construct an efficient system.\textsuperscript{15,16,23,24} Since the rate of the P450 reduction is usually dependent on the electron transfer from its reducing system, construction of a good electron transfer system is essential.

(2) Bioconversion Using Living Cells A bioconversion system using living cells expressing the desired P450 has the following advantages. This system does not require expensive NAD(P)H, and H\textsubscript{2}O\textsubscript{2} produced by the uncoupling reactions (Fig. 1) is scavenged by catalase in the cells. Usually, bioconversion in living cells takes more than 1 d in either growing or resting conditions. Incorporation of the substrates into the cell is often limited, and substrates damaging the cell membrane are unsuitable for this system. Recently, Fujii et al.\textsuperscript{25} constructed genetically engineered E. coli cells deficient in an AcrAB-TolC efflux pump system for the effective production of pravastatin by P450 belonging to the CYP105 subfamily. The enhanced pravastatin production by this system could be explained by the elevated intracellular concentration of the substrate due to the disruption of the efflux pump. This fact indicates the importance of host cell engineering to increase the practical efficacy of P450-dependent bioconversion with living cells.

(3) Bioconversion Using P450 Enzymes as Biocatalysts. (a) CYP102A1 CYP102A1 (P450\textsubscript{hmm,3}) is the most studied P450 and appears to be the most promising P450 as a biocatalyst because it is a soluble, highly active (more than 3000 min\textsuperscript{-1} for the ω-2 hydroxylation of C12 to C18 fatty acid), self-sufficient enzyme (Fig. 2). Based on the structural information obtained by X-ray crystallographic analysis, the numbers of variant molecules of CYP102A1 have been generated by site-directed mutagenesis and/or directed evolution. For example, the F87V variant showed increased activity towards aromatic compounds.\textsuperscript{26} Glieder et al.\textsuperscript{27} have successfully produced alcohols from C3-C8 alkanes by the CYP102A1 variant with 11 amino acid substitutions generated by directed evolution. Peters et al.\textsuperscript{28} have generated CYP102A1 variants which catalyze regio- and enantioselective alkane hydroxylation. These results suggest that protein engineering of CYP102A1 is quite promising for the production of various compounds, including bulk chemicals. However, natural CYP102A1 requires expensive NADPH as the electron donor (Fig. 2). Therefore, it is difficult to use CYP102A1 variants as the biocatalysts for producing bulk chemicals. Recently, Arnold’s group has generated variants of the P450 domain of CYP102A1 that can act with hydrogen peroxide by directed evolution (Figs. 1, 2). Further efforts on the directed evolution of the P450 domain generated thermostable and/or highly active variants.\textsuperscript{29–31} Although this shunt system with the engineered P450 domain prepared by directed evolution appears to be hopeful, elevation of the affinity to hydrogen peroxide is necessary to prevent destruction of the heme group by high concentrations of hydrogen peroxide.

(b) Shunt System If the P450 reaction required neither NAD(P)H nor electron transferring proteins, the application range of P450 would be dramatically expanded. Although most P450s can use peroxides such as hydrogen peroxide, cumene
hydroperoxide and tert-butyl hydroperoxide as the active oxygen sources, this reaction along a so called “shunt” pathway is inefficient in most P450 species, prohibiting their practical use (Fig. 1). However, Bacillus subtilis CYP152B1 (P450_{Bac}) and CYP152A1 (P450_{asp}) and CYP152A2 (P450_{cla}) are natural peroxygenases that require no electron donors and efficiently catalyze α- or β-hydroxylation towards long-chain fatty acids by using hydrogen peroxide. These peroxygenase P450s appear to be promising for practical application in producing hydroxylated products of long-chain fatty acids. Recently, Shoji et al. reported enantioselective styrene epoxidation and ethylbenzene hydroxylation by using short-chain carboxylic acids as decay molecules. By changing the structure of a decay molecule, catalytic activity and enantioselectivity would be improved. A combination of this technology and protein engineering could generate useful biocatalysts that accept a wide range of substrates.

(c) Increase of Stability of P450 The heme molecule of P450 is readily removed under conditions causing a conformational change of P450, and the stability of most P450s is not sufficient for producing chemical compounds on an industrial scale. However, recently Maurer et al. reported that immobilized CYP102A1 showed a half-life of 29 d at 25°C, while the half-life of its free form was 2 d. This finding suggests that a combination of the fusion technology producing P450 with its redox partner and the immobilization technology of the fused enzyme may be useful in generating active and stable biocatalysts. A nanodisc system containing lipids and membrane scaffold protein is a versatile tool for the study of membrane proteins, and is considered a useful method to increase both the activity and stability of membrane bound P450s. Therefore, utilization of a nanodisc containing both P450 and its reductase as a biocatalyst is hopeful possibility.

2. APPLICATION OF P450S TO BIOSENSORS

Efforts to develop a direct electron transfer system to P450 have been made for more than 30 years. Direct reduction of P450 with suitable electrodes has been reported to be useful for drug discovery and development. Joseph et al. developed a biosensor based on the redox properties of CYP3A4. The enzyme films were assembled on gold electrodes by alternate absorption of a CYP3A4 layer on top of a polycation layer. Addition of CYP3A4 substrates caused a concentration-dependent increase in the reduction current in cyclic voltammetric experiments. These results suggest that this biosensor can identify drugs or drug candidates acting as substrates or inhibitors of CYP3A4. Recently, Mie et al. demonstrated that electrochemically-driven drug oxidation was detected by voltammetry by using an electrode coated with hydrophobic thin films and intact microsomes. This method requires no purified enzyme preparations, and the electrode can be prepared easily at a low cost. Determination of the plasma concentration of a drug is usually done with GC-MS or LC-MS, but these methods are time-consuming and expensive. A P450 biosensor response to specific drugs may be a promising alternative that would give quick measurements of drug levels at a low cost. This method might also be applicable to measuring endogenous substances metabolized by P450, such as cholesterol, 25-hydroxyvitamin D₃, and fatty acids.

3. APPLICATION OF P450S TO BIOREMEDIATION

Bioremediation technologies for soils and sediments contaminated with industrial chemicals, such as dioxins and PCBs, have been developed. Angular dioxygenase, P450, lignin peroxidase, and dehalogenase are all known as dioxin-metabolizing enzymes. Our previous studies revealed that mammalian P450s are capable of the degradation of mono-, di-, and tri-chlorobenzo-p-dioxin (CDD). In particular, CYP1A1, CYP1A2, and CYP1B1 showed remarkable activity toward low-chlorinated PCDDs. However, they showed no detectable activity towards 2,3,7,8-TCDD. Thus, we attempted to generate a 2,3,7,8-TCDD-metabolizing P450 by enlarging the space of the substrate-binding pocket of rat CYP1A1, and succeeded. White rot fungus P. chrysosporium has been shown to possess biodegradative capabilities toward DD, 2,7-DCDD, and 2,3,7,8-TCDD. Since the P. chrysosporium genome contains 148 P450 genes, it is possible to assume that some P. chrysosporium P450s can metabolize PCDDs. Recently, we obtained 120 clones expressing individual P450s of P. chrysosporium and six of them could metabolize 2-MCDD. Although CYP5145A3 showed the highest activity among them towards 1-MCDD, 2-MCDD and 2,3-DCDD, no detectable activity was observed toward 2,7-DCDD and higher-chlorinated dioxins. Judging from these results and the tertiary structure of the substrate-binding pocket of human CYP1A2, P450s belonging to the CYP1 family appear to have the most suitable structures for the metabolism of flat polycyclic aromatic hydrocarbon PCDDs, PCDFs, and coplanar polychlorinated biphenyls (PCBs). Although genetically engineered microorganisms (GEMs) have potential risks affecting the biological environment, recent progress in generating suicidal genetically engineered microorganisms will make it possible to apply GEMs expressing suitable P450 for the bioremediation of soil contaminated with PCDDs and PCDFs in the near future.

CONCLUSION AND FUTURE PROSPECTS

At the present time, practical applications of P450 to the biocconversion process as biocatalysts are limited. The reasons limiting the practical applications of P450 are low stability, low activity, and co-factor dependency of most P450s. Extensive efforts have been carried out to overcome these problems in order to expand the application of P450s as biocatalysts and biosensors. I believe that P450s could be much more widely used on an industrial scale in the near future.

Although I have not described it in this review, the most sensational industrial application of P450 is the invention of a blue rose. It is noted that a literary meaning of “blue rose” is “impossible.” Introduction of the flavonoid 3’,5-hydroxylation (CYP75A) gene of viola into a rose made it “possible” to generate a blue rose. A similar application of P450 is the generation of a herbicide-resistant plant expressing mammalian P450s. One plant has more than a few hundred P450 genes that may be responsible for the synthesis of plant hormones and secondary metabolites. Insects have more than 60 P450 genes in their genome which play important roles in hormone synthesis and detoxification of phytalexins and insecticides. It is noted that most fungi have more than 100 P450 genes in their genome, and actinomycetes generally have more than 20
P450 genes, most of which appear to be involved in the synthesis of antibiotics. Now we can utilize P450 gene libraries from all biological kingdoms consisting of a huge number of P450 species. In addition, we can create novel P450s by site-directed mutagenesis and/or directed evolution. Therefore, I believe that the P450 superfamily has tremendous potential for practical applications in various fields.

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REFERENCES


