Protein engineering of oxygenases for biocatalysis

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Oxygenase enzymes have seen limited practical applications because of their complexity, poor stabilities, and often low catalytic rates. However, their ability to perform difficult chemistry with high selectivity and specificity has kept oxygenases at the forefront of engineering efforts. Growing understanding of structure–function relationships and improved protein engineering methods are paving the way for applications of oxygenases in chemical synthesis and bioremediation.

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Abbreviations

BM-3	cytochrome P450 BM-3
BPDO	biphenyl dioxygenase
BphC	2,3-dihydroxybiphenyl 1,2-dioxygenase
CPO	chloroperoxidase
CPR	cytochrome P450 oxidoreductase
HbpA	2-hydroxybiphenyl 3-monooxygenase
NDO	naphthalene dioxygenase
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
pNCA	*p*-nitrophenoxy-carboxylic acid
TDO	toluene dioxygenase
TOM	toluene *ortho*-monooxygenase

Introduction

Oxygenase enzymes comprise several protein families that introduce one (monooxygenases) or two (dioxygenases) oxygen atoms into their substrates. Figure 1 lists examples of oxygenase-catalyzed reactions that are relevant to biocatalysis. Oxygen is typically supplied as O_2, and the required reduction equivalents are usually derived from NADH or NADPH via electron-transfer proteins (e.g. reductase). Collectively, the oxygenases catalyze highly regioselective and stereoselective reactions on a wide range of substrates.

Hydroxylation and epoxidation reactions catalyzed by monooxygenases (Figure 1) are of particular interest in chemical synthesis. The cytochrome P450 monooxygenases comprise a versatile superfamily of enzymes that catalyzes these and other oxidative reactions on substrates that range from alkanes to complex endogenous molecules such as steroids and fatty acids. P450s have been the subject of numerous engineering studies aimed at understanding their functions and properties as well as making better catalysts (reviewed in [1]). Dioxygenases are multimeric, non-heme iron proteins that primarily oxidize aromatic compounds (Figure 1) and therefore have applications in environmental remediation. The chiral *cis*-dihydrodiol products of the dioxygenase reaction are also potentially useful intermediates for natural-product syntheses [2]. A number of excellent dioxygenase engineering studies have been done by Furukawa and co-workers, and Furukawa has reviewed his and others' work [3,4]. Peroxidases have historically been of interest for their ability to catalyze one-electron oxidations of organic substrates [5]. However, peroxidases, and particularly the fungal enzyme chloroperoxidase (CPO), also exhibit selective monooxygenase ('peroxygenase') activity, with peroxide (Figure 1). CPO is thus also an attractive target for protein engineering. Synthetic applications of CPO have recently been reviewed [6].

Demands for clean, economical oxidation processes and for increasingly complex and specific oxidation products provide a strong driving force for considering biocatalytic routes. This review describes recent protein engineering efforts aimed at overcoming the shortcomings of oxygenases, including complexity, poor stability, low expression, or low activity on unnatural substrates.

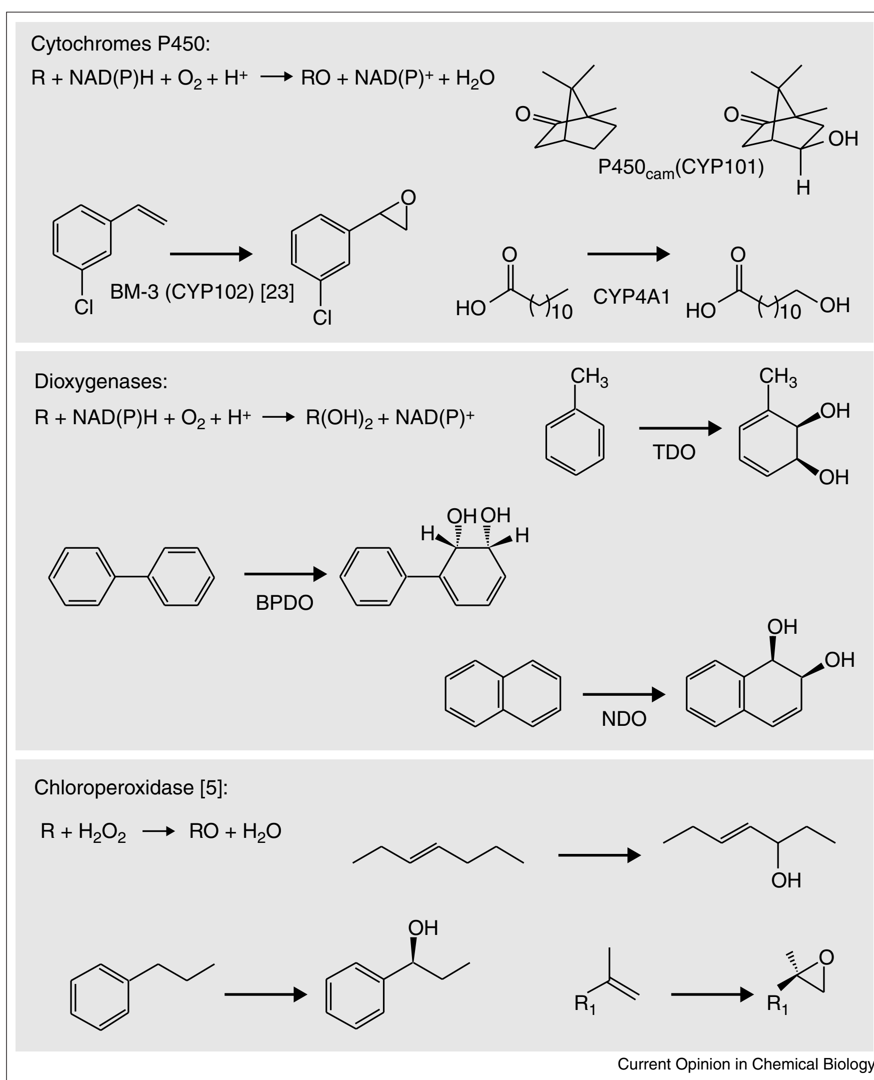
Monooxygenases

Cytochrome P450 BM-3

Cytochrome P450 BM-3 (BM-3; CYP102) from *Bacillus megaterium* hydroxylates long-chain fatty acids at subterminal positions. It is called a 'self-sufficient' P450 because it contains the required diflavin NADPH-P450 reductase on the same polypeptide as the P450 hemoprotein [7]. BM-3 is soluble, easily expressed in recombinant *Escherichia coli*, and highly active, with turnover rates in the thousands per minute for fatty acids [8,9]. A clever colorimetric assay for hydroxylation activity has facilitated several recent engineering studies on BM-3, particularly those needing high-throughput activity screens. The assay, developed by Schwaneberg *et al.* [10], uses *p*-nitrophenoxy-carboxylic acid (pNCA) as a fatty acid surrogate substrate, which upon hydroxylation at the terminal carbon produces yellow *p*-nitrophenolate. Subsequent modifications to the assay have allowed its use in high-throughput screening of enzyme libraries [11] and for alkane substrates [12].

BM-3 residue Phe87 plays an important role in determining the regioselectivity of fatty acid hydroxylation [13]. Substitution with alanine at this position shifts hydroxylation towards the ω position and also increases the sensitivity of the *p*-nitro-phenoxydodecanoic acid (12-pNCA) assay compared with that of the wild-type BM-3 [10]. Using this mutant and the pNCA assay, Schwaneberg *et al.* [14•] found that BM-3 accepts electrons directly from the mediator cobalt(III)-sepulchrate, thereby eliminating the NADPH cofactor. The mediator could be regenerated using zinc dust. BM-3 mutants Phe87Ala and Phe87Gly are also more efficient utilizers of hydrogen peroxide in

Figure 1



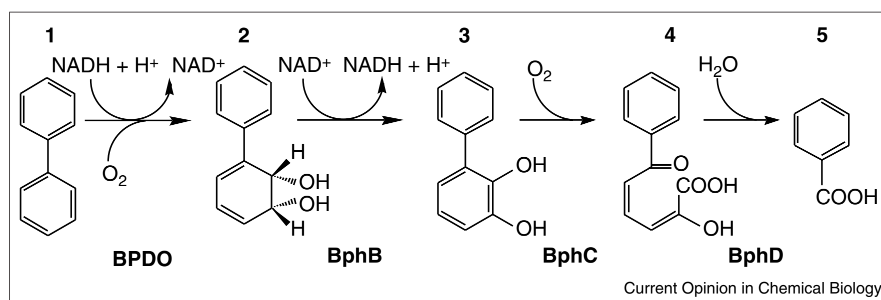
Examples of oxygenase-catalyzed reactions. The overall reaction equation is indicated for each group of enzymes, where 'R' represents substrate.

place of NADPH and O_2 to drive catalysis via the peroxide shunt pathway (peroxygenase activity) [15].

Schmid and co-workers [16–19] combined site-directed mutagenesis with site-specific saturation mutagenesis at residue positions known to affect substrate binding and screened the BM-3 mutant libraries for higher activity on pNCAs with chain lengths of 12, 10, and 8 carbons. They found mutants that accept smaller chain substrates [19], hydroxylate indole [16], oxidize octane and naphthalene much faster [17], and oxidize polycyclic aromatic hydrocarbons (PAHs) poorly accepted by wild type [20]. Carmichael and Wong [21] engineered a BM-3 mutant capable of PAH oxidation by applying site-directed mutagenesis at residues in the active site and at the entrance of the substrate access channel. Both PAH studies report orders of magnitude improvements in activity on different PAHs, although the NADPH coupling efficiencies are all extremely low. Binding and turnover of the small substrates butyrate and hexanoate were greatly increased by generating combinations of site-directed mutations that influence substrate binding [22]. Li *et al.* [23••] demonstrated the importance of residue size at position 87 in determining the stereoselectivity of oxidation of the unnatural substrates propylbenzene and 3-chlorostyrene.

Farinas *et al.* [12] used directed evolution of the BM-3 heme domain to improve activity on saturated hydrocarbons (octane). Random mutant libraries were screened using the substrate analog ω-*p*-nitrophenoxyoctane, which is the alkane equivalent of 8-pNCA. The mutants retained the very high coupling efficiency of the wild-type enzyme on its natural, fatty acid substrates. Further rounds of directed evolution have produced mutants capable of hydroxylating a variety of alkanes (C3 to C8) with turnover rates exceeding those of any known alkane hydroxylase (A Glieder, ET Farinas, FH Arnold, unpublished data). The plasticity of this enzyme is evident and will

Figure 2



Biphenyl catabolic pathway. BPDO oxidises biphenyl (**1**) to 2,3-dihydroxy-4-phenylhexa-4,6-diene (dihydrodiol compound; **2**). Dihydrodiol dehydrogenase (BphB) converts **2** to 2,3-dihydroxybiphenyl (**3**), which is then oxidized by 2,3-dihydroxybiphenyl dioxygenase (BphC) to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (ring *meta*-cleavage compound; **4**). Finally, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase (BphD) produces benzoic acid (**5**). Adapted from [46•].

certainly continue to be exploited in future protein engineering studies.

Other P450s

Wong and co-workers created site-directed mutants of $P450_{cam}$ that oxidize polychlorinated benzenes with considerably enhanced activity and coupling efficiency [24••]. These same mutants were also found to oxidize monoterpenes, which are of interest in fine chemical synthesis [25].

The ability to make functional cytochrome P450–NADPH reductase fusion proteins, particularly for mammalian P450s, would simplify the study and application of these enzymes. Such efforts have been reviewed [1]. Recently, human P450 CYP2D6 has been linked to human NADPH-cytochrome P450 oxidoreductase (CPR), which is the first report of a functionally complete human P450 fusion enzyme system [26]. Site-directed mutagenesis at a single residue converted the human P450 redox partner NADPH CPR to a functional NADH-dependent reductase [27]. Sadeghi *et al.* [28] report a functional fusion between the heme domain of P450 BM-3 and a flavodoxin protein from *Desulfovibrio vulgaris*. Shimizu and colleagues reported that the nitric oxide synthase reductase domain is unable to effectively substitute for that of cytochrome P450 BM-3, whereas the BM-3 reductase domain, in contrast, was able to support low levels of nitric oxide synthase activity [29].

Mammalian P450s are membrane-bound and difficult to express in recombinant organisms, and until recently [30] no crystal structures have been available. Little practical engineering has been done with mammalian P450s; most mutagenesis studies have examined structure–function relationships [31]. Sakaki and Inouye [32] have discussed practical applications of these enzymes. Random mutagenesis methods have recently been applied to eukaryotic P450s [33•,34,35,36•]. DNA shuffling of related sequences is a powerful tool for generating diversity for directed evolution, but it can only be performed on sequences of high identity (>60–70%). Although P450s in general show high structural similarity, sequence identities are often very low, 20% or less [1]. Using a recombination method that does not rely on sequence similarity, Sieber *et al.* [37•] made single-crossover libraries of chimeric P450s and identified enzymes that retained the mammalian parent enzyme's activity, but had improved solubility (characteristic of the parent bacterial enzyme). Sieber's work was inspired by a rationally designed mammalian–bacterial chimera with similar features [38]. Nature's ability to generate functional diversity among human P450 isoforms by alternate usage of exons with very low identity has been demonstrated [39•].

Non-heme monooxygenases

Two non-heme monooxygenases which are capable of hydroxylating aromatic compounds have recently been engineered by directed evolution [40,41]. 2-Hydroxybiphenyl 3-monooxygenase (HpbA) catalyzes the regioselective *ortho*-hydroxylation of a wide range of 2-substituted phenols to the corresponding catechols and has been used for the production of 3-substituted catechols [42]. Meyer *et al.* [40] used random mutagenesis to generate HpbA mutants and screened for improved monooxygenase activity on various 2-substituted phenols by the generation of colors indicative of autooxidation by the reaction products. Mutants were isolated with improved activities as well as improved coupling efficiency. Toluene *ortho*-monooxygenase (TOM) converts toluene to methylcatechol in a two-step process; it also oxidizes naphthalene and trichloroethylene, making TOM a potential biocatalyst for bioremediation [43]. Using error-prone DNA shuffling and spectrophotometric screens that detect naphthol or chloride, Canada *et al.* [41] improved TOM's ability to hydroxylate naphthalene and degrade chlorinated compounds.

Dioxygenases

Naphthalene dioxygenase (NDO) is an $\alpha_3\beta_3$ hexamer and is the first Rieske non-heme iron oxygenase for which the three-dimensional structure has been determined [44]. Site-directed mutagenesis at several residues in the active site of NDO resulted in products not formed by the wild type and demonstrated the designability of this enzyme [45]. Biphenyl dioxygenase (BPDO) is an important enzyme for the degradation of polychlorinated biphenyls (PCBs). Figure 2 shows the biphenyl catabolic pathway and some of the enzymes coded for in the *bph* operon. A combination of directed evolution and rational design generated BPDO mutants capable of oxidizing a broadened range of PCBs and other biphenyl-related

compounds [46•]. Mutants were expressed with dihydrodiol dehydrogenase (BphB) and 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), and screening was accomplished by selecting colonies showing a yellow pigment indicative of the ring *meta* cleavage product (compound **4** in Figure 2). In earlier work, this same group used DNA shuffling to evolve BPDO for enhanced PCB degradation [47]. Recently, a number of the laboratory-evolved dioxygenases were reported to exhibit higher activities in monocyclic aromatic hydrocarbon oxidations [48].

Sakamoto *et al.* [49] used random mutagenesis and saturation mutagenesis in an effort to expand the substrate range of toluene dioxygenase (TDO) to include heterocyclic substrates such as 4-methylpyridine. Screening was accomplished using a sensitive liquid-phase Gibbs assay similar to the solid-phase assay reported previously [50]. BphC is an extradiol dioxygenase responsible for ring *meta*-cleavage of dihydroxybiphenyl, formed by biphenyl oxidation by BPDO followed by dehydrogenation by BphB (Figure 2). Random mutagenesis of the *bph*C gene was performed to isolate mutants that more efficiently oxidize 3-chlorocatechol by distal ring cleavage [51]. Variants with enhanced activity on this substrate as well as novel distal cleavage activity on 3-methylcatechol and 2,3-dihydroxybiphenyl were found.

Chloroperoxidase

CPO has broad substrate specificity and does not require NAD(P)H or additional proteins for catalysis. While CPO is potentially attractive for synthetic applications, protein engineering has been hampered by the inability to express the fungal enzyme in a suitable microbial host. Hager and co-workers [52,53] report the ability to express and screen for CPO mutants in the enzyme's natural host *Caldariomyces fumago*. In one study, they improved *p*-nitrostyrene epoxidation activity eightfold over wild type [52] and, in another, indole oxidation activity was enhanced in 40% aqueous *tert*-butyl alcohol [53]. Unfortunately, using this strain to make and characterize CPO mutants is tedious and problematic [54]. Recently Conesa *et al.* [54] reported functional expression of CPO in *Aspergillus niger*, which may help to simplify manipulations on this enzyme.

Conclusions

Nature provides an arsenal of biocatalysts whose capabilities we are learning to exploit and perfect through protein engineering. Where three-dimensional structures are known and previous studies have elucidated the roles of various residues, rational design efforts have proven quite successful. Directed evolution is an established, powerful tool for engineering proteins, particularly when little is known about structure–function relationships. Reports on directed evolution of oxygenases are appearing with increasing frequency. P450s have been designed to oxidize novel substrates and function without requiring biological cofactors, the substrate range of dioxygenases has been expanded, and the peroxygenase activity of CPO has been enhanced. Further investigations into mechanisms, structures and functions of oxygenases remain at the forefront of enzyme research and will continue to provide clues for more fruitful engineering efforts.

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