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Engineering and application of P450 monooxygenases in pharmaceutical and metabolite synthesis

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Cytochrome P450 monoxygenase (P450s or CYPs) allow access to drug metabolites, necessary for approval of new therapeutics in one step, with increased success being demonstrated using bacterial and fungal P450s. Moreover, 12 of the 13 products of the human metabolism of verapamil can be accessed through engineered and chimeric bacterial P450s. These P450s are also used in the synthesis of pharmaceuticals themselves, including the semi-synthetic production of artemisinin in an engineered cell. The integration of new technologies including ultrasound and polyfluorinated hydrocarbon solvents offers an attractive means by the true synthetic potential of ubiquitous P450s can be fully realised.

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Introduction

Cytochrome P450 enzymes are ubiquitous in living systems and perform a number of roles ranging from degradation of carbon sources [1], to biosynthesis of complex natural products [2] and the detoxification of molecules that may be potentially harmful to the cell [3]. They are found abundantly in both prokaryotes and eukaryotes [4,5]. P450 enzymes are a widely studied class and their catalytic activity is well understood. These heme-containing enzymes reduce molecular oxygen, using the cofactor NAD(P)H as a source of electrons. One of the oxygen atoms is then inserted into a substrate derived from a very wide range of structural types and in doing so enables a range of oxidation chemistries such as hydroxylation, epoxidation, dealkylation or oxidation of nitrogen and sulfur atoms [6–8]. The mechanism for these reactions is well described elsewhere [9,10].

The existence of cytochrome P450 enzymes has been known for several decades, but it is only relatively recently that interest in this enzyme class has taken hold and captured the imagination of chemists and biologists alike. In particular, the hydroxylating ability of these enzymes whereby they insert oxygen into sp3 carbon atoms that are otherwise unactivated is very attractive. In performing such hydroxylations, there is frequently the potential to dramatically shorten alternative chemical synthetic routes, and of course avoid the use of oxidising reagents that are expensive and environmentally damaging. Thus, the rewards are potentially high for those who can develop cytochrome P450 oxidative systems and integrate this enzyme chemistry into commercially important molecules, delivering shorter, greener processes [11].

However, the potential of this enzyme class is still some way from being realised, and represents a challenging system to work with [12]. There is a plethora of reasons for this. For a start, many of these enzymes are large and membrane bound, which is problematic for expressing in a microbial host with high protein yields, solubility and good activity [13,14]. Many P450 enzymes are multi-component, for example eukaryotic P450s such as drug-metabolizing human liver P450s and many plant and fungal P450s implicated in secondary metabolism are two component systems, consisting of a membrane-bound P450 domain that relies on another membrane protein, namely a NAD(P)H dependent diflavin [FAD/FMN] reductase (CPR), for supply of the electrons necessary to drive catalysis. The majority of prokaryotic enzymes comprise three components, though some are self-contained and comprise a single polypeptide having all of the necessary oxidative and reductive functionality [10,15].

P450 engineering

Molecular and synthetic biology are becoming powerful technologies in the development of P450 enzymes. Many commercial and academic groups are attracted by the reduced cost and increased success of producing these enzymes recombinantly. There is now a large pool of these enzymes available for screening and detailed studies. The priority is to translate this existing body of knowledge into viable synthetic systems [16•,17,18].

Various protein engineering approaches have been taken to improve the performance of cytochrome P450s. These approaches are illustrated by the P450 BM3 as a

well-studied representative enzyme within the P450 class due to its high solubility and activity when expressed as a recombinant protein in *Escherichia coli*. This particular P450 is derived from *Bacillus megaterium*, and is distinctive in that it only requires NADPH and oxygen to function, and is a self-sufficient enzyme [19]. The structure of this enzyme is known, forming the basis for redesign.

A large amount of engineering has been performed with the BM3 enzyme, and this is extensively reviewed by Whitehouse *et al.* [20••] and Gillam *et al.* [21]. These reviews describe the approaches taken including directed evolution, rational structurally guided mutation, and random approaches such as error prone PCR. The resulting body of research provides numerous examples where activity, stereoselectivity, regioselectivity and substrate specificities have been successfully altered and indeed many cases where they have not, helping to provide an understanding of how changing certain features of the enzyme active site might influence performance [22,23,24•,25,26].

However, the P450 field still presents challenges for state-of-the-art protein engineering approaches. For example, like certain mammalian P450s, P450 BM3 crystallises in a 'precatalytic' conformation in which no substrate atom lies sufficiently close to the heme iron for oxidation to take place [27]. It is thus unclear whether the conformations in which the residues lining the access channel that are depicted are catalytically relevant, complicating the task of engineering the enzyme using this structural information. Most active site residues have nonetheless been subjected to site-specific mutagenesis with a view to clarifying the roles that they play in the catalytic process — typically comparing fatty acid oxidation rates and product distributions given by the generated mutants with those of the wild-type enzyme [28,29].

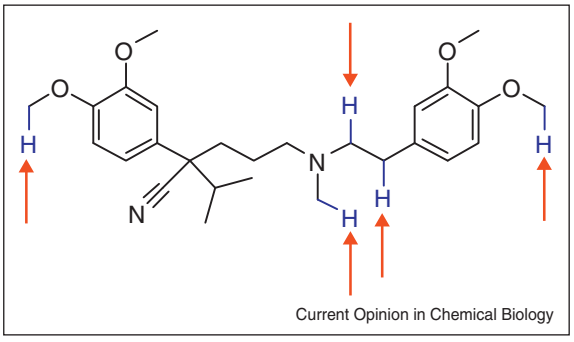
P450 applications

The current demand for P450 chemistry lies primarily within the pharmaceutical field. One particular requirement is for application in metabolite synthesis, where P450s play a naturally prominent role in drug metabolism [30–32]. Characterization of the pharmacological properties, toxicity, and pharmacokinetics of human drug metabolites is a critical part of the drug development process and mandatory for FDA approval of new therapeutics [33]. Thus access to such metabolic products is required, and this presents some serious synthetic challenges arising from the need to devise synthetic routes incorporating the correct stereochemistry and functionality. There is a significant body of literature describing P450-enabled metabolite synthesis, and whilst recombinant human P450s have found some utility, increasing success is being demonstrated with bacterial and fungal P450s [32,34,35]. A good example is published by the Arnold group [36], who demonstrated that 12 of 13 mammalian metabolites of two marketed drugs (verapamil, astemizole) and a research compound (LY294002) could be produced, with varying selectivity and yields, by a panel of 120 engineered and chimeric P450 BM3 variants with broad substrate profile. Figure 1 shows the positions where the panel was able to oxidise verapamil [36].

As well as application in metabolite synthesis, P450 chemistry is also of much interest in the synthesis of pharmaceuticals themselves. Perhaps one of the best examples where current synthetic biology methodology has been applied to P450 enzymes is in artemisinin biosynthesis, where a semi-synthetic route was demonstrated [37•], as illustrated in Figure 2. Amorpha-4,11-diene, a precursor molecule accumulated by an engineered cell, is a challenging target for selective oxidation, where an epoxidation was required for a shorter semi-synthetic route. Wild type P450 BM3 showed no activity towards amorpha-4,11-diene. Subsequently F87 in the substrate binding pocket was identified as a key residue by docking the substrate into the active site of a predicted transition state model. Upon the mutation of this residue to alanine low activity against amorpha-4,11-diene was detected. To increase the size of the binding pocket and, hopefully, improve the yield of epoxide, saturation mutagenesis of key residues in the active site was then preformed. A mutant (F87A, R47L, Y51F and A328L) was identified that was able to accumulate 250 mg/L of the epoxide, which could then be transformed by high yielding chemistry to artemisinin [37•].

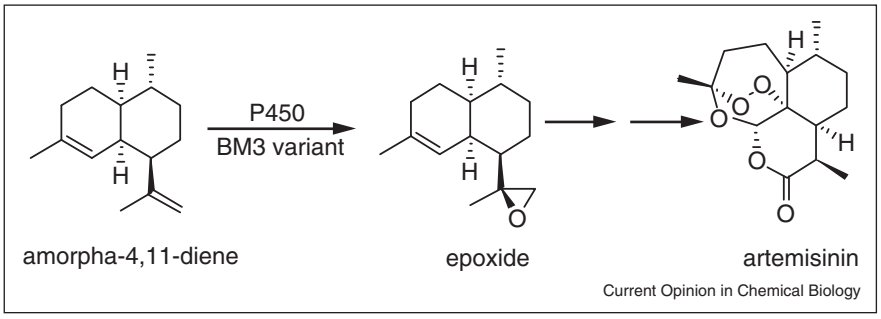
This is an excellent example of how modeling and synthetic biology approaches can be used to redesign P450 enzymes, conceive novel metabolic pathways and provide solutions to challenging chemical problems. As the pool of knowledge increases, particularly relating to structural information, it may be anticipated that this type of approach will rapidly become the method of choice for

Figure 1



Verapamil sites of biooxidation. A panel of engineered and chimeric P450 BM3 mutants were able to oxidise verapamil to produce 12 of the 13 metabolites generated by human P450 metabolism. Oxidation occurred with different regioselectivity as illustrated by the red arrows.

Figure 2



P450 BM3 for artemisinin biosynthesis. P450 catalysed epoxidation of the olefin of amorpha-4,11-diene in an engineered cell. A critical step in the semi synthetic synthesis of the high value anti malarial drug artemisinin.

P450-related chemistry. The increasing number of distinct P450 structures (>30) in the Protein Databank will continue to stimulate the rational redesign and mutagenesis of P450s for improved or altered properties [38].

Future directions

There is ample evidence in the literature of the limitations of P450s with regards to stability, limited turnover, solvent tolerance and substrate specificity. Many attempts have been to try to overcome these problems via protein engineering. In particular, random mutagenesis and the development of chimeric P450 have shown success in improving the thermostability of P450 enzymes whilst retaining the activity of the parental enzyme [39–41]. Other methods have been evaluated for improving stability such as enzyme immobilisation [42]. Protein engineering has proved successful in broadening the substrate specificity, especially the bacterial enzymes which are very substrate specific [20]. These methods are limited by the availability of crystal structure which can aid rational mutagenesis particularly of the active site. The use of organic solvents with P450 has lead to instability and inactivity of the enzymes. The use of random mutagenesis has increased the tolerance of BM3 and CYP 2B1 to organic solvents such as DMSO [43,44].

However, there still exists the need to further develop P450 technology. In our opinion, the future focus for P450 research needs to be on applying new technologies at the molecular level, as elegantly demonstrated above. However, this should also be integrated with methodologies aimed at improving P450 biotransformations at the reaction level, including both physical and chemical approaches.

Future development of P450 reaction systems could investigate and integrate the use of technologies that are known to speed up catalysis in other systems. One such approach may be to utilise ultrasonication, which has been shown to greatly increase the rate of some enzyme reactions, sometimes by an order of magnitude [45–47].

Ultrasound can induce physical phenomena such as cavitation and acoustic streaming, and these lead to extreme conditions of liquid turbulence that can benefit mass transfer, but also influence behaviour at the molecular level such as protein conformation and secondary structure, and lower catalytic activation energies. If applied carefully, beneficial effects such as increased reaction rates, outweigh any negative damaging effects, after all ultrasound is usually used as a destructive tool for protein recovery [48,49••].

A limiting feature of P450 chemistry is the ability to deliver oxygen to the enzyme. Approaches such as use of specialist polyfluorinated hydrocarbon solvents (with surfactants) in which oxygen has much higher solubility could be considered, or reactions performed routinely under higher oxygen pressure [50,51].

Substrate engineering is also a candidate for further development, whereby the substrate is (reversibly) modified [10]. The basis of this approach is to add extra structural functionality to the core molecule to be transformed, whereby the extra functionality serves to help direct and bind the overall molecule in the P450 active site. The core part of the molecule can then be positioned for productive catalysis, and once oxidised, if required, the extra functionality can be removed [52–55].

Conclusions

P450-enabled syntheses are gaining ground, driven by dramatic advances in synthetic biology. The decreasing time and cost of the molecular biology in turn decreases the cost associated with redesign and engineering of P450 enzymes whilst increasing the chances of a successful outcome. However, progress has been and is likely to continue to be slower than other enzyme classes such as ketoreductases and transaminases. In these particular

enzyme classes there have been spectacular success stories — for example, engineering transaminases for sitagliptin synthesis, or ketoreductases for montelukast. P450s are a more challenging enzyme class. Every approach possible needs to be considered, and that includes both redesign of enzymes and how they are applied in biotransformation situations. As high value products, pharmaceuticals will continue to be the main driving force for P450 development, but as examples of larger scale syntheses with P450 emerge we may see increasing interest in fields such as flavour and fragrances. These are higher volume but lower value products, and progress needs to be made before commercially viable processes are possible. It is an exciting field to follow.

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