The power of evolution: accessing the synthetic potential of P450s

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Cytochromes P450 can catalyse hydroxylation reactions that are of considerable potential synthetic value, but a number of practical difficulties have hitherto prevented their use for this purpose. Recent advances, including intelligently designed laboratory evolution experiments, promise to overcome these obstacles, and to add P450s to the enzymatic armoury of the chemist.

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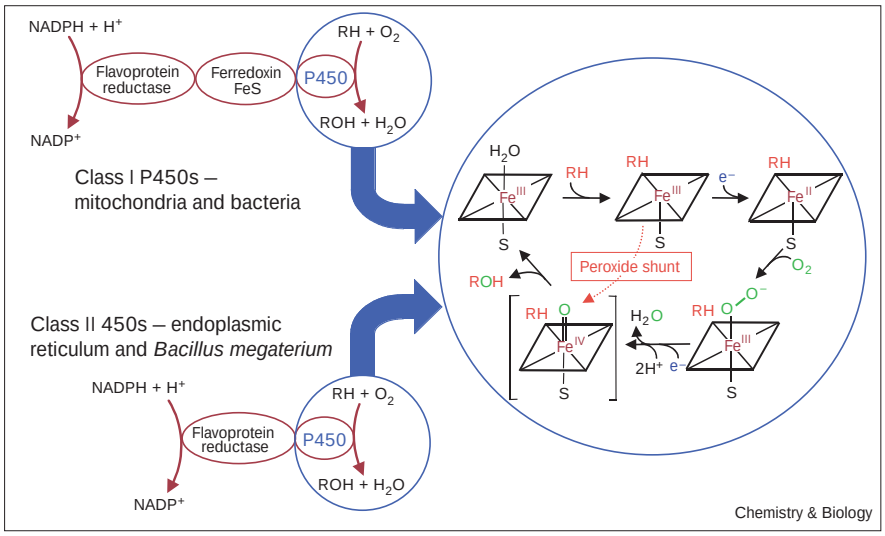
The advantages of enzymes as catalysts of chemical transformations are increasingly widely recognised. Not only do they make it possible to carry out reactions under mild, environmentally friendly conditions — for example in water at room temperature — but they also often show remarkable regio- and stereospecificity. For simple enzymes such as hydrolases, a range of practical applications has already been realised, but other enzymes, notably redox enzymes that require additional protein cofactors for their function, present a greater challenge. A particularly important group of enzymes in this category are the cytochrome P450 monooxygenases (P450s). These ubiquitous enzymes catalyse the insertion of one atom of molecular oxygen into a substrate, with concomitant reduction of the other atom to water. They play essential roles in the biosynthesis of prostaglandins and steroids, as well as of numerous secondary metabolites in plants and microorganisms, and in the detoxification of a wide range of foreign compounds, including both drugs and chemical pollutants. From the synthetic point of view, their ability to hydroxylate unactivated carbons and, for example, their ability to produce pure enantiomers of epoxides are of particular interest. At least 600 P450s are known, with a wide range of substrate specificity, making them, potentially, very attractive synthetic reagents. There are, however, some important obstacles that must be overcome before this potential can be realised: all P450s require at least one redox partner protein, and many of these are membrane-bound and relatively unstable. Several recent papers have reported valuable steps towards overcoming these problems, notably recent reports by Frances Arnold and colleagues [1,2], who describe the use of 'directed evolution' to produce mutants of the bacterial P450cam that catalyse naphthalene hydroxylation using hydrogen peroxide as the source of oxygen, thus bypassing the need for cofactors.

The catalytic cycle of P450s is shown in Figure 1; physiologically, the two electrons required at distinct steps in this cycle are ultimately derived from the nicotinamide coenzymes NADH or NADPH through a flavoprotein reductase. In the monooxygenase system of the mammalian endoplasmic reticulum, for example, which is of crucial importance in drug metabolism ('class II' P450s), electrons are transferred directly from the flavoprotein to the P450. In other cases, including most bacterial P450s and the mammalian mitochondrial enzymes involved in steroid biosynthesis ('class I' P450s), an iron–sulphur protein mediates the electron transfer from the flavoprotein to the P450. Considerable advances have been made in the development of systems for regeneration of the expensive nicotinamide cofactors for application in bio-transformations, but these all involve a further protein and an additional substrate. A system for using a cytochrome P450 in a synthesis therefore appears to involve three or four proteins, together with substrates and cofactors that would need to be separated from the desired product — not an attractive proposition!

In recent years, a number of steps have been taken to simplify this system. First, methods have been developed for the expression of the membrane-bound mammalian enzymes and their purification in the presence of minimal amounts of detergent. Second, it has proved possible to link the components of the monooxygenase system together. This occurs in nature in the form of cytochrome P450 BM3, a bacterial fatty acid hydroxylase that contains both a cytochrome P450 domain and a flavoprotein NADPH-cytochrome P450 reductase domain in a single polypeptide chain [3]. Artificial 'fusion proteins' of this kind have been successfully made for the two-component class II monooxygenases [4] and even for the three-component class I system [5], and shown to have full catalytic activity. We therefore have a single protein able to carry out the monooxygenase reaction, a useful simplification, but we are still faced with the need to supply and regenerate the reduced nicotinamide cofactor. Clearly what is required is to dispense with the need for the cofactor altogether, supplying the electrons in some other way, and Joo *et al.* [1,2] have done so by making use of the so-called 'peroxide shunt' (Figure 1). Addition of hydrogen peroxide (or an organic peroxide) to the initial enzyme–substrate complex supplies both the electrons and the oxygen atom required to form the reactive iron–oxygen intermediate, the species

Figure 1



The electron transfer chains of class I and class II cytochromes P450, and the catalytic cycle common to both classes of enzyme, illustrating the 'peroxide shunt' exploited by Joo *et al.* [1,2].

which inserts the oxygen atom into the substrate. This has the desired result of removing the need for supplying electrons from the redox partner, ultimately from NAD(P)H. The use of peroxide in this way is inefficient, however, and carries with it the danger of inactivation of the enzyme. Although the structure of P450cam, the specific enzyme studied by Joo *et al.* [1,2], has been known for some years [6], too little is known of the mechanistic details to allow the design of mutations to improve the efficiency of this process. It was necessary, therefore, to use random mutagenesis and screening or 'laboratory evolution'.

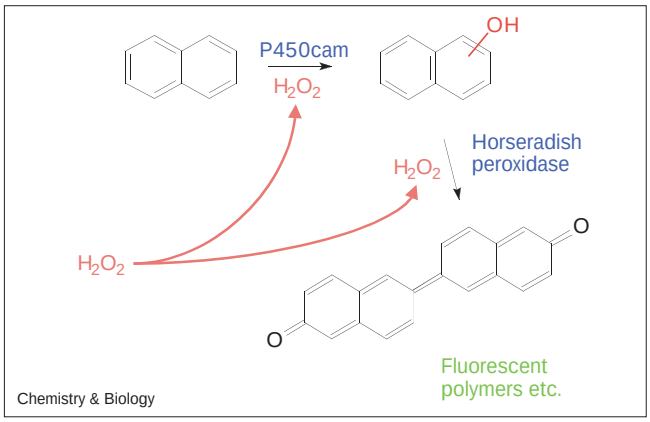
It is well known that the key to success in such an endeavour is the design of an appropriate and efficient screen, and that devised by Arnold and her colleagues [1,2] ingeniously made dual use of hydrogen peroxide, both as substrate and as a detection reagent for the product(s). The objective was to obtain mutants of P450cam able to efficiently hydroxylate naphthalene using the peroxide shunt pathway. The natural substrate of this enzyme is camphor, and the wild-type enzyme shows very little activity against naphthalene, although this can be increased significantly by a single-site mutation in the substrate-binding pocket [6]. The screen used (Figure 2) depended on the fact that the reaction products, monohydroxylated and dihydroxylated naphthalenes, can be oxidatively coupled by horseradish peroxidase (HRP) and hydrogen peroxide to give fluorescent dimers and higher polymers. Fluorescence imaging of a multiwell plate containing colonies of *Escherichia coli* expressing both mutants of P450cam, produced randomly by mutagenic polymerase chain reaction (PCR), and HRP provided, therefore, an efficient means of identifying mutants that had increased activity against naphthalene under these conditions. Approximately 200,000 colonies were screened in this way, and of these about 5,000 showed activity at least as great as the wild-type enzyme. The most active colony identified had 11-fold higher activity than wild type, and also showed greater activity in the assay against the unrelated substrate 3-phenyl-propionate, suggesting that the screen was indeed identifying mutants in which the peroxide shunt is more efficient, as well as those better able to use naphthalene as a substrate. *In vitro* recombination (DNA shuffling — see below) of the genes coding for five of the high-activity mutants identified in the first screen led to a further increase in activity to a maximum of 20-fold greater than that of the wild-type enzyme. An additional benefit of the screen used was that the colour of the fluorescence, reflecting the nature of the polymer formed, in turn reflected the regiospecificity of the initial hydroxylation(s); the range of colours produced clearly showed that many of the mutants generated had regiospecificities different from that of the wild-type enzyme.

There are numerous examples in the literature of the alteration of the substrate specificity, and sometimes the regiospecificity, of cytochromes P450 — not only the bacterial P450cam [7,8] and P450 BM3 [9–11] but also mammalian P450s [12,13] — using site-directed mutagenesis informed by three-dimensional structures or homology models of the enzymes. For example, single residue substitutions in P450 BM3 were able to favour hydroxylation of alkyl trimethylammonium compounds rather than fatty acids [9], and to convert the regiospecificity of saturated fatty acid hydroxylation from (ω–1 + ω–2 + ω–3) to virtually exclusively ω-hydroxylation [10]. Three of the P450cam mutants identified by Joo *et al.* [1,2] in the first round of screening that had at least fivefold greater activity than the wild-type were sequenced. All were found to have a lysine for glutamate substitution at position 133; two had

additional mutations, in one case Arg280→Leu, in the other Cys242→Phe. Residue 242 lies at the edge of the haem pocket, on the proximal side, and it is conceivable that the substitution at this position helps to protect the enzyme from inactivation by peroxide [1]. The other two residues are near, but not in, the substrate-binding pocket, and the origins of the modest effects of these substitutions on catalytic activity are far from clear. This is a salutary reminder of the importance of subtle, long-range effects on enzyme specificity and activity, and of the difficulty in predicting these even in an enzyme as well studied as P450cam. It also demonstrates the power of 'laboratory evolution' to improve the desired characteristics of an enzyme, precisely because it does not depend on the adequacy of our understanding of the contribution of individual residues to determining these characteristics, be they catalytic activity, specificity or thermal stability [14,15].

Two important steps towards the application of P450s in organic synthesis have therefore been taken. First, it is clear that the activity, substrate specificity and regiospecificity of these enzymes can readily be modified by mutagenesis — either by site-directed mutagenesis or by random mutagenesis and screening. Second, the work of Arnold and her colleagues [1,2] offers one route for overcoming the need for a cofactor for these enzymes. What are the prospects for further improvements? The modest increases in activity of 20-fold reported by Joo *et al.* [1,2] were achieved after only two rounds of mutation and selection, and it seems certain that much larger increases will be achievable, notably by further rounds of DNA shuffling. Work by Arnold and by others [16,17] has shown the power of DNA shuffling (*in vitro* recombination) in laboratory evolution. This method involves the generation of chimeric genes from a pool of related genes — for example mutants of the gene of interest — either by fragmentation and reassembly or by a staggered, or interrupted, extension process. Individual mutations are thus 'shuffled' into a wide range of combinations, dramatically increasing the diversity of the population of genes available for selection, and hence the likelihood of obtaining a gene product with the desired characteristics. DNA shuffling can be carried out not only using a pool of mutants of a specific gene, but also using a pool of homologous genes from different species — for example, this approach has been used to speed up dramatically the laboratory evolution of specificity in β lactamases [18]. Given the enormous repertoire of P450 genes encoding enzymes catalysing the same basic reaction but with differing specificity, there would seem to be exciting prospects for altering specificity in this way. For example, one could envisage introducing the specificity of the relatively unstable membrane-bound mammalian enzymes into stable, soluble bacterial enzymes such as P450cam or P450 BM3; the work of Arnold and her colleagues [1,2] is an illustration of the ingenuity that will be required to devise

Figure 2



The basis of the screen developed by Joo *et al.* [1,2], showing the dual use of hydrogen peroxide, both in supplying oxygen and electrons to the P450-catalysed reaction and as part of the product detection system.

appropriate screens. The vast majority of laboratory evolution experiments involve expression of the gene product and selection for the desired activity in a bacterial cell. They are, therefore, likely to be at least much less efficient, if not impossible, in cases where the substrate or the product are toxic to the cell, as might be the case for some organic intermediates; Tawfik and Griffiths [19] have recently described an elegant *in vitro* alternative, however, where an individual gene is transcribed and translated in the aqueous compartment of a water-in-oil emulsion, this cell-like compartment providing the necessary link between the gene and its product to allow laboratory evolution to be carried out.

There are great opportunities for generating P450s with desired catalytic functions. There are also promising alternatives to the peroxide shunt as a means of avoiding the need for a cofactor. Estabrook and his colleagues [20] have shown that it is possible to drive P450 hydroxylation reactions electrochemically using a P450–reductase fusion protein of the kind discussed above, with a platinum electrode and cobalt sepulchrate as a mediator; this has been used successfully for ω-hydroxylation of fatty acids [21] and for the synthesis of 17α-hydroxyprogesterone [22]. More recently, we have shown that electrons can be injected directly into P450s, without the need for the flavoprotein, using a modified gold electrode (A. Abbott, F. Almed, P.M. Cullis, W.U. Primrose and G.C.K.R., unpublished observations; [23]); this has been applied to both P450 BM3 and a number of mammalian P450s. It remains to be seen whether the application of cytochromes P450 in organic synthesis will be commercially viable, but the initial practical obstacles have been overcome, and the prospects are much brighter than they were only a few years ago.

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