Engineering the haem monooxygenase cytochrome P450$_{cam}$ for monoterpene oxidation

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Monooxygenated terpenes are fine fragrance and flavouring chemicals, and active site mutants of the haem monooxygenase cytochrome P450$_{cam}$ which were designed to have improved complementarity between the substrate binding pocket and the monoterpenes (+)-α-pinene (1) and *S*-limonene (2) have been shown to have greatly enhanced activity for the oxidation of these two substrates, and the major products, verbenol and isopiperitenol from (1) and (2) respectively, were formed with high regioselectivity and near-total stereoselectivity.

Terpenes have the general formula $(C_5H_8)_n$ and are biosynthesized from isoprene units.[1] Terpenoid compounds, which mainly comprise the parent terpenes and their oxidation products such as the epoxides, alcohols, aldehydes and ketones, constitute one of largest class of organic compounds in biological systems. Many mono- and sesqui-terpenoid compounds are sought-after fragrances and flavourings due to their distinctive and pleasant odours.[2,3] For example isopiperitenol and carveol, which are intermediates in the biosynthesis of terpenoid compounds that give the characteristic flavours of different species of mint, are formed by the oxidation of (*S*)-limonene by highly specific cytochrome P450 enzymes in the plants.[4,5] As a result of their desirable properties, monooxygenated terpenoids are amongst some of the highest added value fine chemicals. Therefore the one step synthesis of these compounds by direct oxidation of the readily available parent terpenes could have important applications in synthesis. We report here the engineering of the haem monooxygenase cytochrome P450$_{cam}$ for the oxidation of the monoterpenes (+)-α-pinene (**1**) and (*S*)-limonene (**2**).

Cytochrome P450$_{cam}$ catalyses the oxidation of the bicyclic compound D-(+)-camphor to 5-*exo*-hydroxycamphor, the first step in the camphor metabolism pathway of the soil bacterium *Pseudomonas putida*.[6,7] The mechanism, structure, and substrate binding of P450$_{cam}$ have been extensively studied.[8] Since the bicyclic compound (**1**) is structurally related to camphor (Fig.1), we made the reasonable assumption that the two molecules might be bound in similar orientations within the P450$_{cam}$ active site. From the crystal structure the camphor carbonyl group forms a hydrogen bond with Y96 and is in van der Waals contact with F87.[9] Since this carbonyl group is absent from (**1**), the Y96F mutation should improve the binding of (**1**) by removing the polar phenol side-chain,[10] while the larger side-chain of the F87W mutation should improve the enzyme–substrate fit. The C_{10} methyl group of camphor contacts V247. Since the allylic methyl group of (**1**) is in a different position in the molecule, the V247L mutation should also improve the enzyme–substrate complementarity. The mutants Y96F, F87W–Y96F, Y96F–V247L and Y96F–F87W–V247L of P450$_{cam}$ were therefore examined for the binding and oxidation

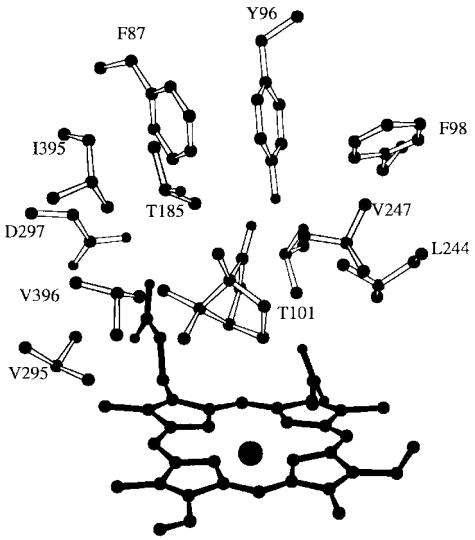

Fig. 1

Table 1 Binding and oxidation of (+)-α-pinene (**1**) and (*S*)-limonene (**2**) by wild-type (WT) cytochrome P450$_{cam}$ and active site mutants

	WT	Y96F	F87W–Y96F	Y96F–V247L	F87W–Y96F–V247L
(+)-α-Pinene (1)					
% High spin haem	85	95	95	95	75
K_D (μM)	1.10	0.15	0.08	0.14	0.30
NADH turnover rate[a]	81.5	147	171	298	129
Product formation rate[a]	18.6	56	96	271	65
Coupling efficiency[c]	23%	38%	56%	91%	51%
(*S*)-limonene (2)					
% High spin haem	20	40	40	60	10
K_D (μM)	5.20	0.55	2.10	1.08	—[d]
NADH turnover rate[a]	4.0	52	100	301	119
Product formation rate[b]	0.2	15.7	31	187	45
Coupling efficiency[c]	5.1%	30%	31%	62%	38%

[a] Given as nanomoles of NADH consumed per nanomole of P450$_{cam}$ per minute. [b] The total amount (in nanomoles) of product formed per nanomole of P450$_{cam}$ per minute. [c] The coupling efficiency is the ratio of the total amount of products formed to the amount of NADH consumed and is expressed as a percentage. [d] Due to the small spectral changes the substrate binding constant could not be reliably determined.

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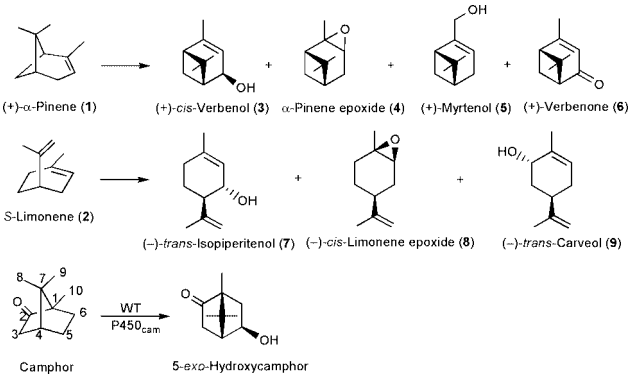

Scheme 1

of (**1**). The monocyclic compound (**2**) could also be bound in a similar orientation to camphor if it adopted the slightly higher energy conformation shown in Fig.1, and these mutants may also have higher activities for the oxidation of (**2**).

The substrate binding and catalytic parameters for the oxidation of (**1**) and (**2**) by wild-type $P450_{cam}$ and the mutants are given in Table 1. The monocyclic (**2**) was a very poor substrate for the wild-type compared to camphor (K_D = 0.25μM, rate = 400 min^{-1} under identical conditions, 100% coupling efficiency). However, the bicyclic (**1**) was bound much more tightly and oxidised at a faster rate with higher coupling than (**2**), in all likelihood reflecting the closer structure of (**1**) to camphor. As predicted the Y96F mutation strengthened the binding and also increased the rate and coupling for the oxidation of both substrates, particularly for (**2**). The addition of the F87W or V247L mutation further enhanced both the binding and oxidation activity for (**1**), suggesting improved enzyme–substrate fits in the F87W–Y96F and Y96F–V247L double mutants. Interestingly (**1**) was bound more tightly by the Y96F and these two double mutants than camphor was by the wild-type, and the fast substrate oxidation rate of 271min^{-1} and near total coupling efficiency of the Y96F–V247L double mutant almost matched the camphor oxidation activity of wild-type $P450_{cam}$. For (**2**) the addition of the F87W and V247L mutations slightly weakened substrate binding but substantially increased the rate and coupling, and so although (**2**) was less tightly bound it was located closer to the haem resulting in more efficient substrate oxidation. The F87W–Y96F–V247L triple mutant showed weaker monoterpene binding and lower activity compared to the double mutants, probably due to steric hindrance between (**1**) and (**2**) and the much smaller active site cavity in this mutant.

Since camphor is selectively oxidised at C_5 to give the *exo* alcohol, the major products from the proposed binding orientation of (**1**) should be (+)-*cis*-verbenol (**3**) and α-pinene epoxide (**4**) (Fig.1, Scheme1). GC co-elution experiments showed that (**3**) was indeed the major product (> 60%) for all the $P450_{cam}$ enzymes. The *cis* and *trans* isomers of (**4**) were minor products (total < 8%), and the enzymes showed little selectivity between the two. In addition (+)-myrtenol (**5**), which arose from oxidation of the allylic methyl group, and verbenone (**6**), the further oxidation product of (**3**), were also formed (Scheme 1). The most active mutant Y96F–V247L gave 70% (**3**) and 7% each of (**5**) and (**6**), while the less active triple mutant F87W–Y96F–V247L was the most selective, giving 85% (**3**).

The selectivity for the oxidation of (**2**) was more sensitive to the mutations. Chiral-phase GC analysis showed that the main products were (−)-*trans*-isopiperitenol (**7**), (−)-*cis*-limonene epoxide (**8**), and (−)-*trans*-carveol (**9**) (Scheme1). All the $P450_{cam}$ enzymes had very high diastereoselectivity (> 95% by GC) for the formation of all these products. Mutants containing the F87W mutation were more selective for (**7**) (82% for both the F87W–Y96F and F87W–Y96F–V247L mutants). The most active mutant Y96F–V247L gave 70% (**7**) but also the highest proportion (16%) of (**9**), while the less active wild-type and Y96F mutant gave the most epoxide (17 and 26% respectively). The predicted major product from the proposed binding orientation was (−)-*cis*-isopiperitenol, and some (*S*)-limonene epoxide was also expected. The observation of the *trans* isomer (**7**) and also some carveol product suggested that the more conformationally mobile (**2**) did not adopt the camphor binding orientation, and that there were multiple substrate binding modes. We note that the P450 enzyme from peppermint oxidises (**2**) with total selectivity to give the *trans*-isopiperitenol (**7**), while the spearmint enzyme gives only *trans*-carveol (**9**).[11,12]

In summary the results suggest that the strategy of designing mutations based on the structure of the monoterpenes and potential side-chain/substrate contacts to improve the enzyme–substrate fit was very successful in promoting monoterpene oxidation by $P450_{cam}$. In addition, with some further selectivity engineering, $P450_{cam}$ variants may have applications in the biotransformation of terpenes in fine chemical synthesis. Finally, since not all of the limonene and pinene oxidation products have been utilised by nature, the oxidation of these and indeed other terpenes by engineered P450 enzymes could give rise to novel fragrances and flavourings or new biologically active compounds.

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