The catalytic activity of cytochrome $P450_{cam}$ towards styrene oxidation is increased by site-specific mutagenesis

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Abstract The styrene oxidation activity of cytochrome $P450_{cam}$ has been greatly improved by rational protein engineering. Compared to the wild-type enzyme, the active-site mutants Y96A and Y96F bound styrene more tightly, consumed NADH more rapidly, and were more efficient at utilising reducing equivalents for product formation. Styrene oxide formation rates were enhanced 9-fold in the Y96A mutant relative to wild-type, and 25-fold in the Y96F mutant, thus demonstrating the effectiveness of active-site redesign in improving the activity of a haem monooxygenase towards an unnatural substrate.

Key words: Monooxygenase; P450; Mutagenesis; Styrene; Epoxidation; Stereoselectivity

1. Introduction

The enzymes of the cytochrome P450 superfamily catalyse many oxidation reactions which are key steps in the biosynthesis and metabolism of steroids, fatty acids and prostaglandins. They also oxidise xenobiotics to more reactive derivatives which may be either easier to remove or more toxic [1–3]. A wide range of molecules, from simple alkanes to steroids, are substrates for P450 enzymes, and individual isozymes can be highly substrate-specific, and selective in the reactions they catalyse. An understanding of the molecular recognition interactions which lead to substrate specificity and catalytic activity is an important step in the redesign of these enzymes to oxidise unnatural substrates, and in the design of inhibitors which may have beneficial therapeutic effects.

The best characterised P450 enzyme is cytochrome $P450_{cam}$ from *Pseudomonas putida*, which catalyses the stereospecific oxidation of camphor to 5-*exo*-hydroxycamphor [1,4]. The oxidation requires dioxygen and two reducing equivalents which are passed from NADH to $P450_{cam}$ via the two electron-transfer proteins putidaredoxin reductase and putidaredoxin. The high resolution crystal structure of camphor-bound $P450_{cam}$ shows that camphor is specifically bound and oriented by a hydrogen bond to the side chain of Y96, and by van der Waals interactions with the side chains of L244, V247 and V295 [5]. Removal of the hydrogen bond by the Y96F mutation [6], as well as mutations of other amino acid residues that line the substrate pocket, e.g. V247, V295, T101 and T185, weakens camphor binding and reduces the specificity of the reaction [7,8].

In addition to camphor derivatives and the closely related tricyclic molecules adamantane and adamantanone, $P450_{cam}$ has been shown to oxidise unrelated compounds such as styrene and its derivatives [9], ethylbenzene [8], nicotine [10], a tetralone derivative [11] and tetrahydronaphthalene [12]. The mechanistic details surrounding the uncoupling of reducing equivalents from hydroxylase activity in ethylbenzene oxidation have been examined in detail [8], and parallel experimental and theoretical studies of the oxidation of both ethylbenzene [13] and styrene [9] have been reported. The wealth of such literature, and the availability of genuine samples of enantiomerically pure metabolites, makes styrene an excellent model substrate on which to base a study of rational redesign of the active site of $P450_{cam}$.

Atkins and Sligar have examined the activity of Y96F towards camphor and analogous compounds such as camphane and thiocamphor [6], and we have shown that $P450_{cam}$ can be engineered by a single mutation, Y96A, to oxidise diphenylmethane which is not attacked by the wild-type enzyme [14]. Here we report the results of styrene oxidation catalysed by wild-type $P450_{cam}$ and these two active site mutants.

2. Materials and methods

2.1. Enzymes

The recombinant forms of cytochrome $P450_{cam}$ and the associated electron-transfer proteins putidaredoxin reductase and putidaredoxin were expressed in *E. coli*, and purified according to literature methods [15–17]. All proteins were stored at −20°C in buffer solutions containing 50% glycerol; the storage buffer for $P450_{cam}$ (40 mM phosphate, pH 7.4) also contained 1 mM camphor. Glycerol and camphor were removed immediately prior to experiments by gel filtration on a Pharmacia PD10 column equilibrated with 50 mM Tris-HCl, pH 7.4.

General DNA manipulations followed standard methods [18]. Site specific mutagenesis was carried out by the Kunkel method [19] according to the Bio-Rad Mutagene Version 2 protocol. A degenerate oligonucleotide was used to generate all possible mutants at the 96 position, and the Y96A and Y96F mutants were identified by DNA sequencing using the Sequenase Version 2 kit from Amersham International.

2.2. Chemicals

All buffer components, solvents and other reagents were of molecular biology grade or the highest purity available. All buffer solutions were filtered through sterile 0.22-μm filters before use. Styrene, ethylbenzene, and *R*- and *S*-styrene oxide were obtained from Aldrich, bovine liver catalase was from Sigma, and NADH was from Boehringer Mannheim.

2.3. Spin-shift assays and dissociation constant determination

UV-Visible absorption spectra of 10 μM wild-type $P450_{cam}$ were recorded between 200 and 800 nm at 303 K in 50 mM Tris-HCl, pH 7.4. Reference spectra were obtained in the presence and absence of 4 mM camphor, thus defining the two endpoints in the transition between the predominantly high and predominantly low spin-states. To estimate the shift to high-spin induced by the binding of styrene to 10 μM wild-type, Y96A and Y96F $P450_{cam}$ in Tris buffer, their spectra were recorded in saturating amounts of the substrate (6 μl of a 1.0 M styrene solution in ethanol, for a final nominal concentration of

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approximately 2.4 mM). Spectra were compared to the references, and the relative populations of the spin states were estimated to ±5%.

Dissociation constants were determined according to the method of Peterson [20], by titrating styrene into substrate-free $P450_{cam}$, and monitoring the optical absorbance spectrum from 350 to 450 nm, in particular the increase in absorbance at 392 nm. The value of K_d was obtained from a plot of the fraction of styrene-bound $P450_{cam}$ versus the concentration of free styrene.

2.4. Activity assays

Activity assays were carried out at 303 K in a Cary 1E double beam UV/Vis spectrophotometer equipped with a Peltier temperature control unit (±0.1°C). The incubation mixtures (1.5 ml) contained 50 mM Tris-HCl pH 7.4, 0.05 μM $P450_{cam}$, 16 μM putidaredoxin, 0.5 μM putidaredoxin reductase, 30 μg/ml catalase, 200 mM KCl, and 2 mM styrene substrate. NADH was added to 250 μM to initiate the reaction, and its concentration was monitored at 340 nm ($\varepsilon_{340\ nm} = 6.22\ mM^{-1}\ cm^{-1}$). The spectrophotometer cell holder was equipped with a magnetic stirrer and the incubation mixtures were stirred throughout the reaction.

Duplicate incubations were performed as above, only without the P450 component, to correct for the background NADH consumption rates due to the putidaredoxin-mediated oxidation of NADH.

2.5. Metabolite determination

To determine the coupling of NADH consumption to styrene oxide formation, a known amount of NADH was added to an incubation mixture to initiate the reaction. The incubation was monitored at 340 nm until the NADH had been consumed. The 1.5 ml incubation mixture was then extracted with 150 μl of chloroform by vortexing and the phases separated by centrifuging for 15 min at 4000×*g* and 4°C. The organic layer was removed and analysed on a fused silica DB-1 column (30 m×0.25 mm i.d.) using a Fisons Instruments 8000 Series gas chromatograph equipped with a flame-ionisation detector. The data was analysed by Fisons Chrom-card for Windows package, Version 1.15. The column temperature was held at 45°C for 2 min and then increased to 140°C at the rate of 4°C per min [9]. Under these conditions, the retention times were: styrene 8.0 min, benzaldehyde 9.8 min, phenylacetaldehyde 12.9 min, and styrene oxide 14.2 min; their lower detection limits were 0.5 μM in the 1.5 ml incubation mixture. A calibration curve, which was linear for the range 0–200 μM styrene oxide concentration, was obtained by extracting a solution containing all of the incubation components except styrene, and analysing the extracts by gas chromatography as described above.

The enantiomers of styrene oxide were well resolved on a Cyclodex-B chirality sensitive GC column (30 m×0.25 mm i.d.). Using a 100°C isotherm, the retention times were 14.1 min for *R*-styrene oxide and 14.5 min for *S*-styrene oxide. To increase the accuracy of peak area integrations, the organic extracts from the reactions were concentrated approximately ten-fold under a stream of nitrogen prior to analysis. These concentrated extracts were also analysed on the DB-1 column to detect any minor products.

3. Results

3.1. Haem spin-state shifts and dissociation constants

The results of spin-state shift and dissociation constant assays are summarised in Table 1. The wild-type $P450_{cam}$ showed a relatively small haem spin-state shift (15% HS) and bound styrene relatively weakly ($K_d = 150$ μM). The mutants, on the other hand, showed both considerably larger spin-state shifts (Y96A, 45% HS; Y96F, 55% HS) and tighter binding of the styrene molecule (Y96A, $K_d = 50$ μM; Y96F, $K_d = 110$ μM).

3.2. NADH turnover activity

Peterson et al. have noted the sensitivity of reduced putidaredoxin to air oxidation [16]. In the presence of putidaredoxin reductase, putidaredoxin is able to catalyse the air oxidation of NADH. Since the oxidation of styrene was generally much slower than that of camphor, the rate of this background process was significant when compared to the overall rates of NADH consumption in the fully reconstituted $P450_{cam}$ system. Hence, this background rate was determined from incubations containing all of the components except $P450_{cam}$, and subtracted from overall rates measured for the fully reconstituted system. The results are summarised in Table 2. The wild-type enzyme consumed NADH very slowly, with a turnover rate of 54 min^{-1}. In contrast, the mutants were nearly 6 times as active, with turnover rates of 260 and 310 min^{-1} for Y96A and Y96F respectively.

3.3. Selectivity of styrene oxide formation and coupling to NADH consumption

The oxidation of styrene by the reconstituted $P450_{cam}$ enzyme system gave styrene oxide as the only detectable product. In contrast to previous reports [9], no other styrene metabolites such as benzaldehyde, phenylacetaldehyde, or ring hydroxylation products were observed. Measurement of the rate of styrene oxide product formation indicated that the Y96A mutant was nearly 9 times as active as the wild-type $P450_{cam}$, while the Y96F mutant was 25 times as active (Table 2). However, the NADH consumption rates suggested that the mutants Y96A and Y96F were only approximately 6 times as active as the wild type. Therefore a higher proportion of the NADH molecules consumed in the reactions catalysed by the mutants resulted in the formation of the product styrene oxide, i.e. the reactions catalysed by the mutants were more coupled. Styrene oxidation catalysed by the wild type was 7% coupled to NADH consumption, while the Y96A mutant showed 13% coupling. The Y96F mutant showed a similar NADH consumption rate as Y96A, but the reaction catalysed by Y96F was much more coupled (32%).

The enantiomers of styrene oxide were well resolved on a chirality sensitive GC column. Both wild-type $P450_{cam}$ and the Y96F mutant gave the *R*- and *S*- enantiomers in a ratio of 15:85. This ratio for the wild-type was in close agreement with literature values [9]. In the case of the Y96A mutant, the *R*- and *S*-enantiomers were formed in a 12:88 ratio.

4. Discussion

In the absence of bound substrate, a cluster of six water molecules is found in the active site of ferric $P450_{cam}$ [21]. One of these waters is bound to the haem iron, which is in the low-

Table 1
Binding and oxidation of styrene by the wild type and the Y96A and Y96F mutants of cytochrome $P450_{cam}$[a]

Protein	% High-spin haem	Dissociation constant (K_d)	NADH consumption rate[b]
Wild type	15±5	150±3	54±9
Y96A mutant	45±5	50±4	260±20
Y96F mutant	55±5	110±3	310±10

[a]All parameters are given as mean ± standard deviation for $n > 5$.
[b]Given as (nmole NADH consumed)(nmole of P450 enzyme)$^{-1}$ min^{-1} at 303 K.

Table 2
Activity and coupling of styrene oxidation by the wild type and the Y96A and Y96F mutants of cytochrome $P450_{cam}$[a]

Protein	NADH consumption rate[b]	Styrene oxide formation rate[c]	Coupling (%)	*R*/*S* ratio[d]
Wild type	54 ± 9	4 ± 1	7 ± 1	15:85
Y96A mutant	260 ± 20	34 ± 3	13 ± 1	12:88
Y96F mutant	310 ± 10	100 ± 6	32 ± 2	15:85

[a]All parameters are given as mean ± standard deviation for $n > 5$.
[b]Given as (nmole NADH consumed)(nmole of P450 enzyme)$^{-1}$ min^{-1} at 303 K.
[c]Given as (nmole styrene oxide produced)(nmole of P450 enzyme)$^{-1}$ min^{-1} at 303 K.
[d]Standard deviation was 0.5%.

spin state, and all six are stabilised by hydrogen bonding between neighbours within the cluster. Binding of the natural substrate camphor expels each of these waters from the cavity, including the sixth ligand water, to produce a five-coordinate predominantly high-spin (HS) haem [22]. This spin-state change is accompanied by a shift of the Sorét band to 392 nm and an increase in the haem reduction potential, which enables the electron-transfer protein putidaredoxin to reduce the haem and initiate the $P450_{cam}$ catalytic cycle.

In the oxidation of camphor, thiocamphor and camphane by both wild-type $P450_{cam}$ and the Y96F mutant, a correlation was noted between the extent of the substrate-induced shift to the high-spin form and rates of NADH consumption [6]. This correlation is also evident, but less compelling, in ethylbenzene oxidation catalysed by wild-type $P450_{cam}$ and various mutants [8], and is observed here in the oxidation of styrene by wild-type, Y96A and Y96F mutant $P450_{cam}$ (Table 1). Each of these studies was carried out at a large excess of putidaredoxin relative to $P450_{cam}$. Under these conditions the rates of reaction were limited by the rate of the first electron transfer from putidaredoxin to $P450_{cam}$ [23], which has been shown to increase with increasing percentage of high-spin ferric haem [24]. It appears, therefore, that a simple spin-state shift assay may be an appropriate predictive measure of catalytic activity for novel mutation/substrate combinations, at least in such cases where the rate-limiting step does not change from the first electron transfer.

Both Y96 mutants exhibited large spin-state shifts relative to the wild-type. These shifts (45% and 55% HS) were comparable to those observed for the binding of camphor analogues such as 1-methylnorcamphor and norcamphor (40–50% HS) to the wild-type protein [7], and to the 45% HS content for camphor binding to the Y96F mutant [6]. Being slightly smaller than camphor and decidedly more planar, styrene may not be bound sufficiently close to the haem to effect the displacement of the iron-bound water in the wild-type enzyme. Also, the more hydrophobic nature of the mutant active sites may facilitate the expulsion of water from the active site upon substrate binding. Finally, the effect of alterations in the protein electrostatic field by the Y96A and Y96F mutations should be considered, since recent quantum mechanical calculations have suggested that it may strongly influence the haem spin-state equilibrium [25]. The removal of a polar side-chain from within the active site may have a significant effect in this context.

Styrene was weakly bound to wild-type $P450_{cam}$ (Table 1), the binding was strengthened in the Y96F mutant, and again in Y96A. Much of the strengthened binding in the mutants could arise from the removal of the hydroxyl group of tyrosine to generate a more hydrophobic active site which may interact more strongly with styrene, and also facilitate the expulsion of water upon styrene binding. The reason behind the two-fold tighter binding of styrene to Y96A compared to Y96F is less obvious, but may arise from structural rearrangements within the active site upon removal of such a large aromatic side-chain. Tyrosine-96 lies between two other aromatic residues (F87 and F98) in a region known for its conformational mobility in the presence of sterically demanding inhibitor molecules [26]. Its removal could result in local movements of the surrounding residues, to yield an active site which interacts more favourably with the styrene molecule.

Three potential branch points which could give rise to uncoupling of NADH consumption from substrate oxidation have been noted for the $P450_{cam}$ catalytic cycle [8,27]. The first of these, autoxidation of the ferrous-oxy complex to yield ferric $P450_{cam}$ and superoxide anion, is slow relative to the turnover rate of the catalytic system and does not compete with the second electron transfer to form the ferric-hydroperoxide intermediate. In the second branch, however, the release of hydrogen peroxide from the ferric-hydroperoxide species competes very effectively with cleavage of the O-O bond, a step which generates the putative ferryl intermediate. The final branch partitions this ferryl between hydrogen abstraction from the substrate, thereby generating a substrate radical species which collapses via oxygen-rebound to yield oxidised product and ferric $P450_{cam}$, and further reduction to water via the acceptance of two additional electrons delivered by putidaredoxin.

Freutel et al. have shown that the uncoupling of styrene oxidation catalysed by wild-type $P450_{cam}$ occurs almost exclusively at the second branch point, leading to the formation of hydrogen peroxide [9]. The key determinant controlling uncoupling at this point is thought to be water distribution at the active site, specifically the access of water to the haem-bound oxygen of the ferric-hydroperoxide [8,27], and so we sought to construct a more hydrophobic active site by the removal of the hydroxyl group of tyrosine-96. Indeed, NADH consumption in both Y96A and Y96F mutants was more coupled to product formation than the wild-type enzyme. The nearly threefold lower coupling of Y96A compared to Y96F was not expected, but is qualitatively consistent with a larger hydrophobic pocket in the alanine mutant, rendering styrene less effective at excluding water from the active site during the catalytic cycle. Nevertheless, the combined effect of more rapid NADH turnover and higher coupling resulted in a nearly nine-fold enhancement in the rate of styrene oxide production in Y96A versus the wild-type, and a twenty five-fold enhancement in the case of Y96F.

The oxidation of styrene by wild-type $P450_{cam}$ has been reported to give other products in addition to styrene oxide, namely benzaldehyde, small quantities of phenylacetaldehyde

and a ring-hydroxylated isomer [9], whereas we have observed only the epoxide. Benzaldehyde was shown to arise from the direct oxidation of styrene by hydrogen peroxide generated by uncoupling of the $P450_{cam}$ catalytic cycle. Since catalase was included in our incubations in order to prevent such peroxide-driven reactivity from obscuring the intrinsic activity of the $P450_{cam}$ proteins, benzaldehyde would not have been observed. The absence of phenylacetaldehyde and the ring-hydroxylated isomer remains unexplained, however, since the reported total formation rates of these products (17% of the styrene oxide formation rate) would have generated concentrations which were well within the detection limit under our experimental conditions.

The stereoselectivity of styrene oxide formation was slightly, but significantly, increased by the Y96A mutation (WT and Y96F, 15:85 *R:S* ratio; Y96A, 12:88). This, together with the significantly tighter binding of styrene to Y96A, seems to indicate a subtle change in the binding mode of styrene. It is difficult to account for such small perturbations in the absence of detailed structural data.

In conclusion, we have shown that, at least for a well-characterised enzyme such as $P450_{cam}$, it is possible to develop a rational mutagenic strategy based on straightforward structural and mechanistic reasoning which dramatically improves the binding and turnover activity of an unnatural substrate by a haem monooxygenase enzyme. It should be possible, perhaps with the help of X-ray crystallographic data, to design additional mutations which will further increase the activity and selectivity of styrene oxidation, and this work is in progress.

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