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Short communication

A P450 BM-3 mutant hydroxylates alkanes, cycloalkanes, arenes and heteroarenes

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Abstract

P450 monooxygenases from microorganisms, similar to those of eukaryotic mitochondria, display a rather narrow substrate specificity. For native P450 BM-3, no other substrates than fatty acids or an indolyl-fatty acid derivative have been reported (Li, Q.S., Schwaneberg, U., Fischer, P., Schmid, R.D., 2000. Directed evolution of the fatty-acid hydroxylase P450BM-3 into an indole-hydroxylating catalyst. Chem. Eur. J. 6 (9), 1531–1536). Engineering the substrate specificity of *Bacillus megaterium* cytochrome P-450 BM3: hydroxylation of alkyl trimethylammonium compounds. Biochem. J. 327, 537–544). We thus were quite surprised to observe, in the course of our investigations on the rational evolution of this enzyme towards mutants, capable of hydroxylating shorter-chain fatty acids, that a triple mutant P450 BM-3 (Phe87Val, Leu188-Gln, Ala74Gly, BM-3 mutant) could efficiently hydroxylate indole, leading to the formation of indigo and indirubin (Li, Q.S., Schwaneberg, U., Fischer, P., Schmid, R.D., 2000. Directed evolution of the fatty-acid hydroxylase P450BM-3 into an indole-hydroxylating catalyst. Chem. Eur. J. 6 (9), 1531–1536). Indole is not oxidized by the wild-type enzyme; it lacks the carboxylate group by which the proper fatty acid substrates are supposed to be bound at the active site of the native enzyme, via hydrogen bonds to the charged amino acid residues Arg47 and Tyr51. Our attempts to predict the putative binding mode of indole to P450 BM-3 or the triple mutant by molecular dynamics simulations did not provide any useful clue. Encouraged by the unexpected activity of the triple mutant towards indole, we investigated in a preliminary, but systematic manner several alkanes, alicyclic, aromatic, and heterocyclic compounds, all of which are unaffected by the native enzyme, for their potential as substrates. We here report that this triple mutant indeed is capable to hydroxylate a respectable range of other substrates, all of which bear little or no resemblance to the fatty acid substrates of the native enzyme.

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1. Introduction

CYP102 is a self-sufficient P450 monooxygenase from *Bacillus megaterium* (P450 BM-3) in which the catalytic heme domain and the FAD-containing reductase domain are naturally fused (Li and Poulos, 1997). It has been functionally expressed in *Escherichia coli* and is conveniently prepared and purified in gram quantities (Schwaneberg et al., 1999). Its known function is subterminal, i.e. $(\omega-n)$ hydroxylation of saturated and epoxidation of unsaturated medium- and long-chain fatty acids (Boddupalli et al., 1990; Guengerich, 1991) and its specific activity in this reaction is about 1000-fold higher than eukaryotic P450 monooxygenases of a similar specificity (e.g. CYP52A3 and CYP52A4 from *Candida maltosa*, Scheller et al., 1996). Moreover, *B. megaterium* P450 BM-3 is water-soluble and thus, other than most other members of the P450 family which are multi-protein complexes attached to membranes, can be incorporated in a bioreactor using a zinc-mediator complex instead of expensive NADPH as an external electron donor (Schwaneberg et al., 2000). As the X-ray structure of P450 BM-3 has been resolved at 2.0 Å resolution (Ravichandran et al., 1993), a number of reports have appeared on the putative substrate binding site of this enzyme and have been corroborated by site-directed mutagenesis.

2. Materials and methods

2.1. Enzyme

The P450 BM-3 (Phe78Val, Leu188Gln, Ala74Gly) mutant was obtained as described before (Li et al., 2000).

2.2. NADPH-assay

For the transformations, a 60 mM substrate solution in acetone (50 μl), 50 mM K_xPO_4 buffer (pH 7.5, 9.1 ml), and 2.9 nM (equivalent to 0.05 mg P450, as determined by the carbon-monoxide binding procedure) wild-type or BM-3 mutant enzyme (Li et al., 2000) were combined. For the data reported in Table 1, the mixture was pre-incubated for 5 min, and transformation was started by adding 5 mM aqueous NADPH (300 μl). NADPH consumption was monitored by measuring absorbance at 340 nm. When it had dropped to the blank value, NADPH addition

Table 1
NADPH consumption was measured at 340 nm[a]

Substrates		Reaction rate wild type[b]	Reaction rate mutant[b]
Alkanes, alkanoic acids	Dodecanoic acid	560	788
	Octanoic acid	2.4	35
	Octane	2.5	1760
Alicyclic compounds	α-Ionone	0.8	155
	β-Ionone	1.1	670
Aromatic compounds	Naphthalene	0.8	147
	Anthracene	1.1	45
Heterocyclic compounds	Quinoline	0.9	49
	2-Methylquinoline	1.2	63
	6-Methylquinoline	0.8	58
	8-Methylquinoline	0.9	302

[a] Reaction rates were calculated using an extinction coefficient of NADPH; $\varepsilon = 6.22\ mM^{-1}$.
[b] Reaction rates are given in nmol substrate/nmol P450/min.

was repeated twice (300 μl, 5 mM solution). After complete NADPH consumption, diethyl ether or dichloromethane was added (5 ml), and extraction repeated twice with 5 ml solvent each. The organic phases were combined, dried with $MgSO_4$, and analyzed by thin layer chromatography (TLC, Lutz-Wahl et al., 1998).

2.3. GC-analysis/NMR-analysis

For gas chromatography (GC) and non-magnetic resonance (NMR) analysis, the reaction conditions described above were scaled-up to 10 ml, and NADPH was added in three portions of 100 μl each. GC analyses, Carlo Erba HRGC 4160; on-column injection; column 30 m × 0.25 mm, DB5 (0.25 μm); FID detection. ^{1}H-NMR, Bruker ARX 500, 5 mm dual probe head, $CDCl_3$ solution, 298 K.

3. Results and discussion

The data in Table 1 show indeed that medium-chain alkanes as well as some alicyclic, aromatic, and heterocyclic compounds are good substrates for the BM-3 mutant though not for the wild-type enzyme. Monitoring NADPH consumption is a standard procedure to test for the substrate specificity of P450 monooxygenases (Boddupalli et al., 1990). Since one cannot differentiate, with this experimental setup, between NADPH consumption by productive processes or by side reactions, which leave the substrate unaffected, it is mandatory to prove both structure and amount of any hydroxylation product formed in the course of the enzymatic reaction. For the four most active transformations listed in Table 1, we have complied with this prerequisite by analyzing the reaction mixture extract, after addition of an internal standard, by capillary GLC (with co-injection of authentic standards and/or GC/MS) and, when required, by ^{1}H-NMR.

3.1. Octane (n-alkanes)

On the GLC evidence, octane is transformed by the mutant enzyme almost quantitatively into 2-, 3-, and 4-octanol (molar ratio 1:2.6:2.2, calculated on the basis of the FID response). The chromatogram of the ether extract shows $< 1\%$ residual octane substrate but not even a trace of 1-octanol (all assignments verified by co-injection). The three product peaks in the GLC are identified unequivocally by GC/MS (underivatized, EI mode), with the position of the OH functionality clearly demonstrated by the respective $CH_3(CH_2)_{m,n}CH{=}OH^+$ ions for the two α-fragmentation processes.

3.2. α-Ionone (alicyclic compounds)

Judging from NADPH consumption and TLC analysis, both α- and β-ionone are smoothly oxidized. Detailed GC and GC/MS analyses of the α-ionone hydroxylation extract show 70% conversion, mainly to the two diastereoisomeric 3-hydroxy derivatives (49%) formed by a hydroxylation of the CH_2-group adjacent to the ring double-bond (Lutz-Wahl et al., 1998) and to a product which has been hydroxylated in the butenoyl side chain (17%), besides some other minor hydroxy derivatives ($< 5\%$ in all).

3.3. Naphthalene (aromatic hydrocarbons)

The GLC for the non-concentrated extract shows only the peaks for the substrate naphthalene and 1-naphthol (FID response 10.3:89.7). When the solvent is gently blown off, though, and a ^{1}H-NMR spectrum of the residue recorded in $CDCl_3$, a set of signals appears (besides those for naphthalene and 1-naphthol) which can be clearly assigned to 1,2-dihydro-1,2-dihydroxynaphthalene, i.e. the characteristic dienediol intermediate of an arene dioxygenation (Engesser et al., 1989). The molar ratio of 13.5:64.0:22.5, determined for these three structures by integration, corresponds to 13.5:86.5 between unreacted substrate and hydroxylated derivatives, virtually identical with the value determined from the FID response. The dienediol apparently suffers regiospecific H_2O elimination to 1-naphthol in the course of the GLC analysis.

3.4. 8-Methylquinoline (heteroarenes)

The gas chromatogram of the original ether extract shows two principal peaks (FID response 61:39). The major one is identified as the substrate peak by co-injection of authentic material. GC/MS verifies this assignment, and establishes the structure of the second peak as a hydroxy methyl quinoline. In concentrating the original extract for NMR analysis by gently blowing off the solvent, 8-methylquinoline is partially taken off with the ether, and the hydroxy product fortuitously enriched in the residue. A detailed analysis of the ^{1}H-NMR spectrum shows that the three-spin system of the heterocyclic moiety is conserved in the hydroxy product down to the coupling fine structure. While 2-H and 3-H are shifted minutely relative to 8-methylquinoline ($+0.7_0$ and -2.1_5 Hz, respectively, i.e. 0.001_4 and -0.004_3 ppm at 11.74 T), 4-H experiences an extreme downfield shift of 0.399 ppm. This is compatible only with the OH substituent in 5-, i.e. in the peri-position. The larger $^3J_{ortho}$ coupling constant (7.6 Hz) for the two remaining protons in the carbocyclic moiety constitutes additional proof for the 5-hydroxy structure. There is evidence from the NMR spectrum also of isomeric substitution products, though in very minor quantities ($< 5\%$ each).

A surprisingly wide substrate specificity, quite contrary to that of the native enzyme, thus has been demonstrated for the triple BM-3 mutant towards aliphatic, alicyclic, and aromatic hydrocarbons as well as towards aza-heteroarenes, though with a strong structural dependence for each specific substrate. Naphthalene, for instance, is hydroxylated, with high efficiency and regioselectivity, to 1-naphthol. Anthracene, on the other hand, is virtually not accepted, and neither is pyridine, 9H-carbazol, or quinoline while 8-methylquinoline is again transformed efficiently.

Surprisingly, n-octane is oxidized at a similar rate as n-dodecanoic acid, a preferred substrate of both the native enzyme and the triple mutant, and about 20-fold faster than octanoic acid. It remains to be seen how n-octane, which lacks any functional group for specific substrate binding, is efficiently oxidized in an (ω–n) fashion which closely parallels the (ω–n) oxidation of fatty acids by native P450 BM-3. The enantioselectivity, in contrast, appears severely impeded, while the native enzyme produces exclusively (ω–n)-hydroxy fatty acids with *R* configuration, alkane hydroxylation by the BM-3 mutant, from our preliminary evidence, gives an *R*/*S* mixture of (ω–n) hydroxyalkanes, although with at least a 2:1 preference in favor of the *R* enantiomers. Incidentally, this new BM-3 mutant was evolved expressly for the oxidation of shorter-chain fatty acids; it oxidizes octanoic acid in (ω–n) position — a reaction which does not take place with wild-type P450 BM-3 (Shao and Arnold, 1996).

For the alicyclic, aromatic, and heterocyclic compounds tested so far, oxidation by the BM-3 mutant is not fully regioselective, though often one regioisomer is formed preferentially, e.g. 1-naphtol from naphtalene or 5-hydroxy-8-methylquinoline from 8-methylquinoline. A computer model of the P450 BM-3 mutant shows all three mutations, which were evolved in a random procedure using saturated mutagenesis (Lentz et al., 2001) to be in or close to the substrate binding pocket. The model gives no indication, though, how these mutations actually influence the unusual catalytic behavior of our BM-3 mutant. This aspect is now being investigated by QSAR and Fe-NMR studies.

The triple BM-3 mutant, obtained by serendipity, may not be the only example of P450 BM-3 mutants with an unusual specificity in substrate oxidation. In view of the potential of P450 monooxygenases for the regio- and stereoselective hydroxylation of non-activated carbon–hydrogen bonds and of the unique advantages of a recombinant P450 BM-3 system we are presently investigating how this enzyme, and mutants thereof, can be exploited in the chemical synthesis of various organic chemicals.

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