Screening of a minimal enriched P450 BM3 mutant library for hydroxylation of cyclic and acyclic alkanes†

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A minimal enriched P450 BM3 library was screened for the ability to oxidize inert cyclic and acyclic alkanes. The F87A/A328V mutant was found to effectively hydroxylate cyclooctane, cyclodecane and cyclododecane. F87V/A328F with high activity towards cyclooctane hydroxylated acyclic *n*-octane to 2-(*R*)-octanol (46% ee) with high regioselectivity (92%).

Effective oxidation of readily available cyclic and acyclic alkanes provides compounds which find applications in manufacturing soaps, detergents and solvents, as well as in the production of certain raw materials. For example, cyclododecanol and cyclododecanone are applied in the synthesis of polyamides and macrocyclic lactones.[1] The important chiral intermediates such as (*R*)- or (*S*)-2-octanol are widely used as versatile synthons and precursor compounds for the preparation of pharmaceuticals, agrochemicals, pheromones and liquid crystals.[2] However, the controlled oxidation of the notoriously inert alkanes is still a challenging task for modern chemistry and biotechnology.[3]

Regarding oxidation of acyclic *n*-alkanes of different chain lengths several heme- and non-heme iron monooxygenases have been reported, which hydroxylate alkanes exclusively at ω-position.[4] In the area of protein engineering numerous mutants of CYP101A1 from *Pseudomonas putida* (P450cam) and CYP102A1 from *Bacillus megaterium* (P450 BM3) have been constructed for oxidation of *n*-alkanes ranging from decane to ethane, yielding, however, mixtures of secondary alcohols.[5] The highest regioselectivity towards 2-octanol of 82–89% was reported by Arnold and colleagues for the P450 BM3 mutants with multiple (up to 17) mutations, which produced either (*R*)-enantiomer (39% ee) or (*S*)-enantiomer (65% ee), correspondingly.[6]

With respect to cyclic alkanes several reports describe degradation of cyclohexane either by single bacterial strains of *Nocardia*, *Pseudomonas*, *Acinetobacter* and *Xanthobacter* or by cyclohexane-degrading consortia.[7] The pathway proposed for the degradation of cycloalkanes includes the initial ring hydroxylation by a monooxygenase, followed by formation of the corresponding ketone by a dehydrogenase, and finally by the insertion of one oxygen atom into the ring, catalyzed by a Baeyer–Villiger monooxygenase.[8] A previous study describes the strain *Rhodococcus ruber* CD4 that is able to oxidize cyclododecane and the corresponding monoketone with subsequent ring cleavage like that during cyclohexane degradation.[7] Whereas numerous Baeyer–Villiger monooxygenases have been isolated and characterized,[10] almost nothing is known about the corresponding cycloalkane hydroxylases. Previously we reported a P450 BM3 mutant that hydroxylates cyclohexane.[9] Degradation of cyclic alkanes with a higher C-atom number, like cyclodecane and cyclododecane are far less investigated. Thus, there is still a demand in active (and selective) alkane hydroxylases.

In the present report a minimal enriched P450 BM3 mutant library was tested with four substrates of different shape, size and chemical reactivity: cyclooctane (C8), cyclodecane (C10), cyclododecane (C12). This library was previously constructed by combining five hydrophobic amino acids (alanine, valine, phenylalanine, leucine and isoleucine) in two positions, 87 and 328, located directly above the heme-group (Fig. 1). The library contains 24 variants, 11 of those demonstrated a strong shift in regio- or stereoselectivity during oxidation of different terpenes and terpenoids.[11]

P450 BM3 is a self-sufficient fusion protein, consisting of a heme domain and a diflavin reductase domain. For its activity the enzyme requires only the pyridine cofactor NADPH. All 24 P450 BM3 mutants were actively expressed in *E. coli*, purified and tested with target compounds. The wild type enzyme converted only 8% of cyclooctane and 3% of cyclodecane with low activity (Table 1). Cyclododecane was not accepted by wild type P450 BM3 at all. The screening of the mutant library revealed 20 out of 24 mutants which were able to convert cyclooctane; 17 mutants accepted cyclodecane as substrate and 8 mutants were able to oxidize cyclododecane.

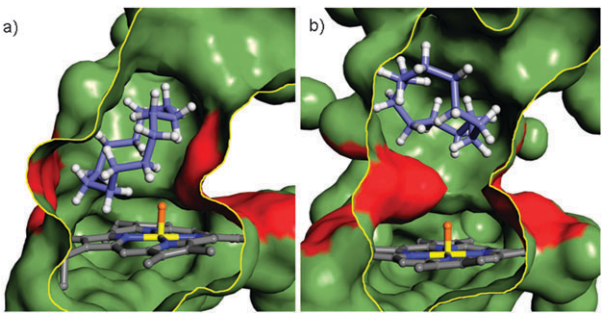

Fig. 1 The substrate binding cavity of P450 BM3 F87A/A328V (a) and P450 BM3 wild type (b) in complex with cyclododecane after 3 ns of unrestraint MD simulation. The mutated positions are depicted in red. Positions 87 (left) and 328 (right) stabilize the substrate in the active site cavity. The activated oxygen of the heme is shown in orange.

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Table 1 Oxidation rates[a] and conversions[b] of three cyclic alkanes measured with selected P450 BM3 mutants[c]

	Cyclooctane		Cyclodecane		Cyclododecane	
Enzyme	Ox. rates (eq. min^{-1})	Conv (%)	Ox. rates (eq. min^{-1})	Conv (%)	Ox. rates (eq. min^{-1})	Conv (%)
WT	2	8	<1	3	—	—
F87A	20	65	13	57	4	20
F87A/A328F	130	90	23	60	1.5	6
F87A/A328I	102	85	28	70	3.6	14
F87A/A328L	141	75	78	50	—	—
F87A/A328V	87	80	20	60	18	46
F87V	46	70	67	60	2.4	6
F87V/A328F	230	75	35	14	—	—
F87V/A328I	73	82	16	23	—	—
F87V/A328L	64	52	20	19	—	—
F87V/A328V	34	58	13	23	1	3
A328V	200	87	106	53	—	—

[a] Oxidation rates given in nmol product per nmol P450 per min (referred to as eq. min^{-1}) were measured by GC/MS after 15 min. Reactions contained 1 μM P450, 100 μM NADPH, 200 μM substrate in 2% DMSO and potassium phosphate buffer, pH 7.5. [b] Conversions in % were measured with 1 μM P450, 600 μM NADPH and 200 μM substrate after 1 h. [c] All experiments were performed in triplicates on two independent occasions. Errors are not higher than 8%.

In all the cases the single oxidation product was the corresponding alcohol. The detailed GC-analysis revealed, however, that in many cases conversion of cyclic substrates was less than 20%. Only 6 mutants converted >70% of cyclooctane to cyclooctanol; 5 mutants demonstrated >50% conversion of cyclodecane to cyclodecanol, and at least 2 were able to convert >20% cyclododecane. In some cases higher initial oxidation activity did not lead to higher conversions, which might be due to lower mutants' stability.

The detailed analysis of all P450 BM3 mutants, which resulted in more than 20% conversion with at least one of the substrates (Table 1), has revealed that most of them contain alanine or valine at position 87, independent on the substrate tested. However, the mutants containing F87V resulted generally in lower conversions of cyclodecane and cyclododecane compared to cyclooctane. Only the F87A/A328V mutant was able to oxidize the largest substrate cyclododecane with a relatively high activity of 18 nmol product per nmol P450 per min and resulted in 46% conversion. Interestingly, it was not sufficient to solely increase the accessibility of the heme by replacing the bulky residues in the hotspot positions by smaller amino acids to maximize conversion of these large substrates as indicated by the higher conversions by the mutant F87A/A328V compared to the F87A. Obviously, the mutant with the better fitting shape of the substrate binding cavity can stabilize the substrate more effectively during catalysis.

The relatively large size of cyclododecane prompted us to study by molecular dynamics simulations how this substrate is accommodated in the active site cavity of the most active mutant F87A/A328V in comparison to the wild type enzyme. The mutations F87A and A328V were introduced to the structure of P450 BM3 (PDB entry 1bu7A) with the Pymol 0.99 program.[12] The cyclododecane molecule was placed at a distance of 6 Å to the activated heme oxygen by hand. The complex was simulated in explicit water using the Amber 8 molecular dynamics simulation program package.[13] The system was coupled to an external pressure and temperature bath.[14] The force field ff03 was used and the partial charges of cyclododecane were taken from corresponding atom types in amino acids. During the time course of the simulation the substrate approached the heme in mutant F87A/A328V and remained stable in close proximity to the heme centre with the closest C–H···O–Fe distance of 2.95 Å. A fluctuating root mean square deviation of the protein backbone between 1.0 and 1.2 Å from the crystal structure further indicates that there is no major impact by the substrate on the protein backbone. The cyclododecane molecule is in close contact with the amino acid side chains of the two hotspot positions (Fig. 1a). Interestingly, the small side chain of alanine in the mutant F87A/A328V creates a space close to the heme which is occupied by the substrate. In the wild type enzyme this space is occupied by a more bulky side chain of phenylalanine in this position (Fig. 1b). As a consequence the activated oxygen is less accessible for the substrate. This observation might explain why the double mutant converts cyclododecane, while the wild type does not.

A recent report on degradation of cyclododecane by the *R. ruber* CD4 strain suggests that the larger the carbon ring of the cyclic alkane, the more flexible it is and the easier it can take a conformation like that of an open-chain alkane. This observation was also made during docking of cyclododecane. Since fixation of inert alkanes in the binding pocket of P450 BM3 occurs almost exclusively *via* hydrophobic interactions, and as there is no "terminal" part of a ring, the reaction with acyclic alkanes can be considered as "analogous" to a subterminal attack on an acyclic substrate.[7] Consequently, a preference for larger rings therefore may indicate a good potential for attack at a certain subterminal position on a linear aliphatic structure. Our previous calculation of activation energy barriers for H-abstraction of different positions of *n*-octane revealed a slightly lower activation energy for ω-1 position (corresponding to 2-octanol) compared to all other positions.[15] Based on both the hypotheses we tested the same set of the P450 BM3 mutants with *n*-octane for improved regioselectivity towards 2-octanol formation.

The P450 BM3 wild type enzyme demonstrated very low activity and regioselectivity upon *n*-octane hydroxylation. After 30 min reaction substrate conversion achieved *ca.* 5%, which correlates with the previously reported data.[16] The ratio of 2-octanol in the product mixture was 15%. Besides that 3-octanol (37%), 4-octanol (43%) as well as 5% ketones were

Table 2 Oxidation rates[a] and conversions[b] of *n*-alkane with selected P450 BM3 mutants[c]

		n-Octane	
Enzyme	Ox. rates (eq. min^{-1})	Conversion (%)	Ratio of 2-octanol (%)
WT	4	5	15
F87A	5.5	15	12
F87A/A328F	5.2	8	49
F87A/A328I	7.2	13	60
F87A/A328L	18	10	25
F87A/A328V	9.1	10	7
F87V	9	5	20
F87V/A328F	22	15	92
F87V/A328I	11	13	51
F87V/A328L	15	14	85
F87V/A328V	12	12	47
A328V	144	33	67

[a] Oxidation rates given in nmol product per nmol P450 per min (referred to as eq. min^{-1}) were measured by GC/MS after 15 min. Reactions contained 1 μM P450, 100 μM NADPH, 200 μM substrate in 2% DMSO and potassium phosphate buffer, pH 7.5. [b] Conversions in % were measured with 1 μM P450, 600 μM NADPH and 200 μM substrate after 30 min. [c] All experiments were performed in triplicates on two independent occasions. Errors are not higher than 10%.

formed. Eight out of 24 mutants from the minimal library produced 2-octanol in a ratio of >25%. The strongest shift in regioselectivity towards 2-octanol (product ratio of >50%) was observed with mutants, containing a bulky amino acid at one of the two hotspots, if the second one was represented by a smaller alanine or valine residue. When both positions 87 and 328 were occupied by valine and/or alanine, the ratio of 2-octanol dropped to 7% (Table 2).

The best mutants regarding 2-octanol production were F87V/A328L (85%), F87/A328V (single mutant, 67%) and F87V/A328F (92%). Probably, the flexible *n*-octane molecule should be "fixed" more tightly, for example by the bulky phenylalanine or isoleucine either at position 87 or 328, in order to be oxidized preferably at one position. The similar correlation between structural changes in the substrate binding pocket and enzyme regioselectivity upon *n*-octane oxidation was observed previously for the homologous CYP102A3 from *B. subtilis*.[17]

The same mutants, F87V/A328L, F87/A328V and F87V/A328F demonstrated high conversions of cyclooctane, significant conversions of cyclodecane, but low or no conversion of cyclododecane. These results suggest that high activity towards a cyclic alkane may indeed indicate a potential specificity for a subterminal attack on a corresponding acyclic structure. However, no direct correlation can be built without additional experiments on other cyclic and acyclic substrates. Moreover, in all the cases the more flexible *n*-octane was converted much slower than the cyclic substrates (Table 2).

Investigation of the enzyme enantioselectivity demonstrated that the most regioselective mutant F87V/A328F produced up to 92% of (*R*)-2-octanol with 46% ee. In comparison, the wild type enzyme produced 15% (*R*)-2-octanol with 20% ee. The P450 BM3 mutant that produced up to 82% of (*R*)-2-octanol (39% ee) as reported by Peters *et al.*, contained 13 mutations, including A328V.[6a] Though being less regioselective than the here reported F87V/A328F, that mutant demonstrated higher activity. This confirmed our suggestion that both positions 87 and 328 in combination control enzyme regioselectivity, while all other mutations influence mainly enzyme activity and stability.

In summary, several P450 BM3 mutants were identified which enable oxidation of C8–C12 cycloalkanes. To our knowledge this is the first example of active cycloalkane's monooxygenases. Furthermore, our results demonstrate the functional flexibility of P450 BM3 mutants with minimal number of mutations. Single and double P450 BM3 mutants with substitutions in the active site were engineered, accepting and hydroxylating inert alkanes to corresponding alcohols, and in the case of acyclic alkanes—with high regio- and moderate enantioselectivity.

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