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Regio- and stereoselectivity of P450-catalysed hydroxylation of steroids controlled by laboratory evolution

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A current challenge in synthetic organic chemistry is the development of methods that allow the regio- and stereoselective oxidative C–H activation of natural or synthetic compounds with formation of the corresponding alcohols. Cytochrome P450 enzymes enable C–H activation at non-activated positions, but the simultaneous control of both regio- and stereoselectivity is problematic. Here, we demonstrate that directed evolution using iterative saturation mutagenesis provides a means to solve synthetic problems of this kind. Using P450 BM3(F87A) as the starting enzyme and testosterone as the substrate, which results in a 1:1 mixture of the 2β- and 15β-alcohols, mutants were obtained that are 96–97% selective for either of the two regioisomers, each with complete diastereoselectivity. The mutants can be used for selective oxidative hydroxylation of other steroids without performing additional mutagenesis experiments. Molecular dynamics simulations and docking experiments shed light on the origin of regio- and stereoselectivity.

Oxidative C–H activation of complex organic compounds with regio- and stereoselective introduction of hydroxy functions at predetermined non-activated sites (C–H → C–OH) is a challenging problem in synthetic organic chemistry[1–3]. Following Breslow's early work on photochemically mediated oxidation of steroids[4], other types of template-based strategies using transition metals have been described, in which functional groups already present in a simple or complex compound are used for directing the position of oxidative attack or for directing which supramolecular factors are exploited[2,5–7]. In some cases, the electronic properties of very simple reagents, in the absence of directing groups, transition-metal catalysts or ligands, dictate the chemical outcome[3,8–10], examples being the dioxiranes, which preferentially hydroxylate the sterically least shielded tertiary C–H centres in steroids[8] and other compounds[3,9]. Catalytic processes have also been developed[2,11,12]. Further current progress includes the use of rigid Fe-complexes[13] and bulky divanadium-substituted phosphotungstate polyoxometalates[14] as catalysts in regioselective H_2O_2-mediated C–H oxidation of non-natural compounds.

Enzymes are one alternative to synthetic reagents and catalysts[15]. In nature, cytochrome P450 monooxygenases (CYPs) catalyse the O_2-mediated hydroxylation of complex compounds such as terpenes, alkaloids or steroids at late stages of biosynthesis, in many cases with high selectivity[15–18]. However, if 'foreign' lipophilic compounds enter organisms, detoxication by way of solubilization resulting from oxidative C–H hydroxylation need not be regio- or stereoselective. In some (industrial) applications, scientists have screened collections of P450 containing microbial strains with the discovery of selective systems for steroid hydroxylation[8,15–18]. Nevertheless, such an approach is not uniformly successful, because it is difficult to consistently reach >95% regio- as well as >95% stereoselectivity. Protein engineering studies[19,20] of P450 enzymes as catalysts in the oxidative hydroxylation of complex compounds have been reported[21–24], including those focusing on steroids[25–29] using, for example, alanine scanning[25,26], but none has achieved this goal. Indeed, the development of techniques that allow such ideal transformations at arbitrarily selected sites on an optional basis, in a steroid or any other organic compound, remains a challenge[30]. Here, we show that knowledge-guided directed evolution[19,20] based on saturation mutagenesis in the form of the iterative Combinatorial Active-Site Saturation Test (CAST)[20] constitutes a tool for solving this synthetic problem. Sites comprising one or more amino-acid residues next to or near the binding pocket are first randomized with the formation of focused libraries that are screened for activity and/or selectivity. An improved mutant at one site is then used as a template to perform saturation mutagenesis at another site, and this 'Darwinian' process is repeated until the desired degree of catalyst improvement has been reached[20].

We chose P450 BM3(F87A)[16–18,24,31,32] as the biocatalyst, which is a mutant of wild-type (WT) P450 BM3 (CYP102A1) found in *Bacillus megaterium*[33]. This enzyme and the mutant are expressable on a gram scale in *E. coli*[34] and have one of the highest CYP turnover rates[35]. P450 BM3 consists of a natural fusion between haem-Fe-dependent monooxygenase and the electron-delivering NADPH-dependent diflavin-reductase. Crystal structures of the haem domain of WT P450 BM3 in the unbound[36] and substrate-bound[37] forms are available. Although the natural substrates of WT P450 BM3 are believed to be fatty acids, this particular mutant was originally designed to influence regioselectivity[31]. Subsequently, it and other mutants with 'smaller' residues at position 87 were shown to accept a number of non-natural substrates[32], the mutations reducing steric congestion at the active haem-Fe site[31,32,38].

In the present study, the oxidative hydroxylation of testosterone (**1**) was chosen as the model reaction, a substrate not accepted by WT P450 BM3[27]. In contrast, we discovered that P450 BM3(F87A) is active, but it leads to a 51:46 mixture of the 2β- and 15β-hydroxylated products **2** and **3**, respectively. The remaining 3% are other regioisomers comprising 6β- and 16β-hydroxytestosterone. Our goal was twofold: to evolve a mutant of P450 BM3(F87A) that ensures >95% regioselectivity in favour of 2β-hydroxytestosterone (**2**),

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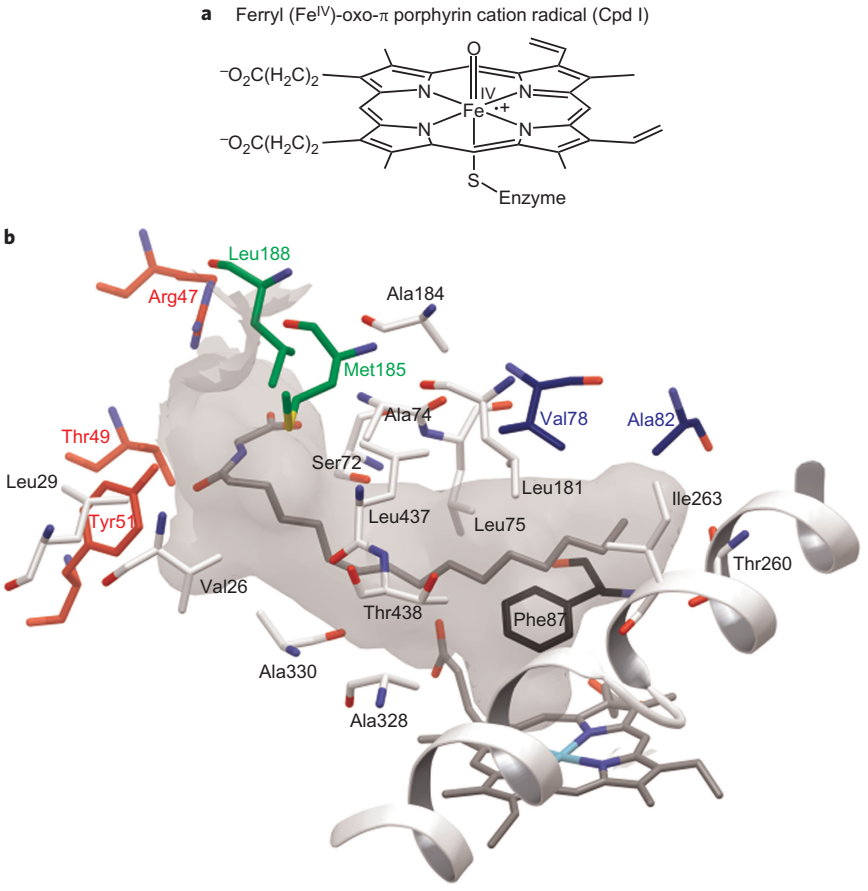


Figure 1 | Mechanism- and structure-based choice of sites for saturation mutagenesis. a, Structure of the catalytically active high-spin haem-Fe species (Cpd I)[16–18,39,40], which initiates oxidative C–H activation by abstracting a hydrogen atom from the bound substrate. **b**, Overview of the utilized CAST sites A (red), B (dark blue) and C (green). Shown is the *N*-palmitoylglycine (dark grey) bound WT P450 BM3 (1JPZ)[37], with residue Phe87 in black. Residues from further CAST sites are marked in white. Haem-complexed iron is shown in light blue. Helix I is given as a point of reference. A more detailed description of the sites can be found in the Supplementary Information.

and to obtain a different mutant displaying opposite regioselectivity leading to >95% in favour of 15β-testosterone (**3**). Erosion of diastereoselectivity (α/β selectivity) or significant shift to alternative hydroxylation sites had to be avoided. We believed this work could lay the foundation for an emerging set of mutants as part of a toolbox that organic chemists can use for regio- and stereoselective hydroxylation of steroids.

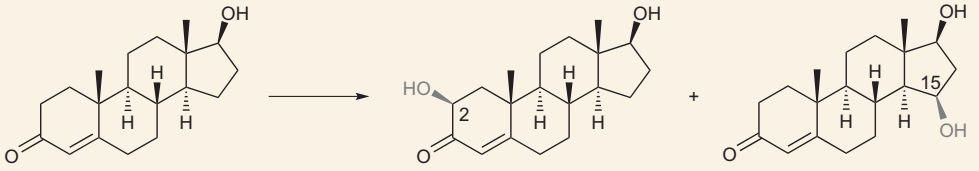
Results and discussion

Saturation mutagenesis experiments. To choose appropriate CAST sites for saturation mutagenesis, mechanistic and structural data were used. It is well known that the mechanism of P450-catalysed C–H activation involves the intermediacy of a high-spin oxyferryl porphyrin radical cation (Cpd I) (Fig. 1a), which abstracts a hydrogen atom from the bound substrate, followed by C–O bond formation of the short-lived alkyl radical[16–18,39,40]. The crystal structure of P450 BM3 in complex with *N*-palmitoylglycine[37] was used as a rough guide for identifying 20 residues as possible candidates for mutagenesis, in spite of the possibility that repositioning of the substrate may occur prior to reaction[38]. Grouping amino-acid residues into two- or three-residue sites maximizes the probability of cooperative effects (more than additivity) within a given site and between sites as the evolutionary process proceeds[20]. We therefore grouped the 20 residues into nine sites, A (Arg47, Thr49, Tyr51), B (Val78, Ala82), C (Met185, Leu188), D (Ser72, Ala74, Leu75), E (Leu181, Ala184), F (Thr260, Ile263), G (Ala328, Ala330), H (Leu437, Thr438) and I (Val26, Leu29). In initial experiments we focused on three sites: A, B and C (Fig. 1b). We also considered residues Ser72, Ala328 and Ala330, using them as single amino-acid sites to minimize the screening effort at this stage of the investigation. Automated high-performance liquid chromatography (HPLC) was used for medium-throughput screening, with increased activity and selectivity being the criteria for identifying hits.

Conventional NNK codon degeneracy encoding all 20 canonical amino acids in saturation mutagenesis at a two-residue site would require, for 95% library coverage, the screening of ~3,000 transformants, which increases to 1×10^5 when choosing a three-residue site[20]. A reduced amino-acid alphabet, as defined by the respective codon degeneracy, was therefore considered, which we have found previously to be effective in the directed evolution of other enzymes[20]. In the case of sites A and C we opted for NDC codon degeneracy encoding only 12 amino acids (Gly, Val, Leu, Ile, Phe, Ser, Cys, Tyr, His, Asp, Asn and Arg), which is a balanced mixture of polar/nonpolar, charged/non-charged and aromatic/non-aromatic residues. This reduced set requires the screening of only 430 transformants for 95% coverage of a two-residue site. The two-residue site B was randomized using NNK codon degeneracy. The screening of the three initial libraries involved a total of 8,700 transformants. Table 1 summarizes some of the results, highlighting in each library typical mutants having increased activity and moderate to high selectivities.

As a general trend, the 2β-selective mutants originate from library A, and 15β-selective variants are found mainly in library B. It can be seen that, even with these initial saturation mutagenesis

Table 1 | The best mutants obtained by saturation mutagenesis at sites A, B, C and residue Ala330 using the gene of P450 BM3(F87A) as template.



Mutant	Library	Mutations	% Conversion*	2	:	3	:	Others†
Starting enzyme	—	F87A	21	52		45		3
KSA-1	Lib 330NNK	A330W/F87A	79	97		3		0
KSA-2	Lib A	R47I/T49I/Y51I/F87A	67	94		6		0
KSA-3	Lib A	R47I/T49I/Y51V/F87A	53	94		6		0
KSA-4	Lib A	R47Y/T49F/F87A‡	84	37		62		2
KSA-5	Lib B	V78L/A82F/F87A	86	3		91		6
KSA-6	Lib B	V78T/A82F/F87A	58	4		91		6
KSA-7	Lib B	V78I/A82F/F87A	78	3		89		8
KSA-8	Lib B	V78I/A82D/F87A	69	84		16		0
KSA-9	Lib B	V78V/A82N/F87A‡	32	58		40		2
KSA-10	Lib B	V78C/A82G/F87A	55	44		55		1
KSA-11	Lib C	M185N/L188N/F87A	65	28		68		4
KSA-12	Lib C	M185S/L188C/F87A	74	49		50		1
KSA-13	Lib C	M185G/L188G/F87A	73	47		52		1
KSA-14	Lib AB	R47Y/T49F/V78L/A82M/F87A§	85	3		96		1
KSA-15	Lib AB	R47Y/T49F/V78I/A82M/F87A§	91	3		94		3
KSA-16	Lib AB	R47Y/T49F/V78T/A82F/F87A§	60	2		90		8

*Conditions: 1 mM testosterone, 24 h, 25 °C, resting cells containing expressed P450 mutants. For detailed expression and reaction conditions, see Supplementary Information. †Minor side products were identified by HPLC as 16β-hydroxytestosterone and 6β-hydroxytestosterone. ‡Wild-type amino acid was retained in at least one position; mutant KSA-9 contains a silent mutation V78V (GTA → GTG). §Mutant KSA-4 was used as a parent and site B was addressed. Mean values are given, standard deviation for conversion is ±5% and for selectivity ±1%, and complete data sets are included in the Supplementary Information. Differences from 100% occur from rounding.

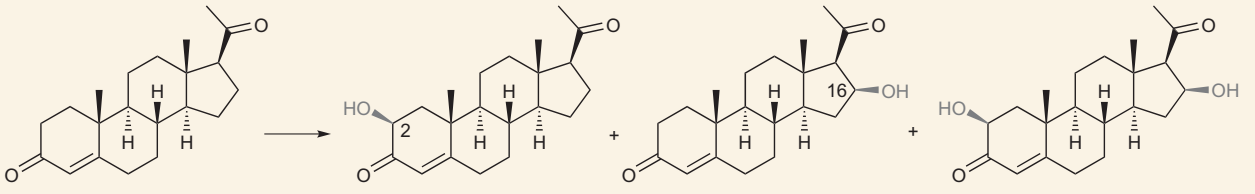
libraries, the synthetic goal we had set was essentially reached. For example, mutant KSA-2 originating from library A displays 94% regioselectivity in favour of the 2β-product **2**, and no erosion of diastereoselectivity or increased formation of other regioisomers are observed. Interestingly, the single mutant KSA-1 from library 330NNK proved to be even better (**2**:**3**:other = 97:3:0). Reversal of regioselectivity in favour of **3** is likewise possible, for example, by using mutant KSA-5 from library B, which delivers a product ratio according to **3**:**2**:other = 91:3:6. Library C was found to harbour mainly 15β-selective mutants, albeit with moderate regioselectivity (Table 1). The improved mutants showing the highest regioselectivity are also among the most active ones.

Thus, this appears to be one of the cases in which the initial mutagenesis libraries harbour enzyme mutants that essentially solve the catalytic problem under study without having to apply iterative rounds of saturation mutagenesis (ISM)[20]. We nevertheless applied this technique to further optimize 15β-selectivity, a process for which several options are available. Rather than subjecting the gene of the best mutant from library B to saturation experiments at site A or C, we chose the most active 15β-selective mutant from library A, namely KSA-4 (Table 1), which is less selective than hits of library B, and used its gene as a template for randomization at site B. In this curiosity-driven experiment, we knew from previous work regarding the directed evolution of an enantioselective enoate-reductase that the use of 'not the best' mutant in an ISM process may in fact be highly successful or even superior[41]. We wanted to test this in the present case, while keeping the screening effort at a minimum (see Supplementary Information). Following the screening of 600 transformants, several improved variants were identified, the best (KSA-14) having a regioselectivity of **3**:**2**:other = 96:3:1 The newly introduced mutations are Val78Leu and Ala82Met, which means that mutant KSA-13 is characterized by a total of five mutations (R47Y/T49F/V78L/A82M/F87A).

Other steroidal substrates. It was of interest to test some of the evolved mutants as catalysts in the hydroxylation of other steroids such as progesterone (**4**), without performing any additional mutagenesis experiments. The starting enzyme P450 BM3(F87A) was found to be active, but delivers an 18:82 mixture of the 2β- and 16β-products **5** and **6**, respectively, with no sign of the dihydroxylated product **7**. Thus, the change in substrate shifts the reaction at position 15 to position 16, but 16β-regioselectivity is mediocre, and clean hydroxylation at the 2-position is not at all feasible. Gratifyingly, high regioselectivity in favour of **5** or **6** on an optional basis proved to be possible using the appropriate mutants (Table 2). For example, the optimal 2β-selective mutant is KSA-9 (A82N/F87A), which displays 100% regioselectivity, and the best 16β-selective mutant is KSA-1 (F87A/A330W), resulting in the reversal of regioselectivity (**5**:**6**:**7** = 9:91:0).

Kinetics and coupling of product formation with NADPH consumption. As the screening step was designed to identify mutants showing enhanced activity (in addition to improved selectivity), we expected to detect this effect quantitatively upon performing kinetic analyses. Using testosterone (**1**) as the substrate, kinetic parameters for the starting enzyme P450 BM3(F87A) and several 2β- and 15β-selective mutants were obtained by measuring NADPH consumption. Table 3 shows that the mutants are indeed considerably more active towards this substrate than the starting enzyme, the effect being particularly drastic in the case of variant KSA-14, with a 152-fold increase in product formation rate. Moreover, whereas in the case of P450 BM3(F87A) the efficiency of coupling of product formation with NADPH consumption amounts to only 6.5%, the 2β- and 15β-selective mutants (KSA-2 and KSA-14, respectively) lead to coupling values of 30% and 46%, respectively. Thus, directed evolution has provided viable catalysts in terms of activity, selectivity and efficiency. Increased coupling of product formation

Table 2 | Oxidative hydroxylation of progesterone (4) using P450 BM3 mutants evolved previously for the conversion of testosterone (1).



4 5 6 7

Mutant	Library	Mutations	5	:	6	:	7	:	Others*
Starting enzyme	—	F87A	18		82		0		0
KSA-1	Lib 330NNK	A330W/F87A	9		91		0		0
KSA-4	Lib A	R47Y/T49F/F87A†	23		63		12		2
KSA-5	Lib B	V78L/A82F/F87A	26		51		12		12
KSA-7	Lib B	V78I/A82F/F87A	30		59		3		8
KSA-9	Lib B	V78V/A82N/F87A†	100		0		0		0
KSA-10	Lib B	V78C/A82G/F87A	13		81		4		3
KSA-14	Lib AB	R47Y/T49F/V78L/A82M/F87A‡	11		64		22		4
KSA-15	Lib AB	R47Y/T49F/V78I/A82M/F87A‡	8		75		14		4
KSA-16	Lib AB	R47Y/T49F/V78T/A82F/F87A‡	57		37		0		6

Conditions: 1 mM progesterone, 24 h, 25 °C, resting cells containing expressed P450 mutants. For further expression and reaction conditions, see Supplementary Information. *Traces of side products were identified as 17α, 21-dihydroxyprogesterone and 21-hydroxyprogesterone by HPLC. †Wild-type amino acid was retained in at least one position; mutant KSA-9 contains a silent mutation V78V (GTA → GTG). ‡Mutant KSA-4 was used as a parent and site B was addressed. Mean values are given, standard deviation for conversion is ±5% and for selectivity ±1%, and complete data sets are included in the Supplementary Information. Differences from 100% occur from rounding.

Table 3 | Kinetic parameters and coupling efficiency: product formation and NADPH consumption during testosterone hydroxylation.

Mutant	Selectivity	K_M (mM)	k_{cat} (s^{-1})	n_H	V_{max} (min^{-1})*	CE (%)†	R (s^{-1})‡
F87A	2β, 15β	0.098 (±0.0233)	0.31 (±0.01)	—§	—§	6.5 (±0.10)	0.042
KSA-2	2β	1.378 (±0.0640)	—§	1.35 (±0.02)	18.6 (±3.6)	30.0 (±0.90)	0.780
KSA-8	2β	0.065 (±0.0255)	1.36 (±0.08)	—§	—§	11.0 (±0.35)	0.403
KSA-5	15β	0.021 (±0.0029)	1.57 (±0.12)	—§	—§	42.4 (±1.33)	1.221
KSA-14	15β	0.016 (±0.0004)	6.64 (±0.57)	—§	—§	45.8 (±1.60)	7.636

Kinetic parameters were obtained measuring NADPH consumption; see Supplementary Materials and Methods. For coupling of product formation to NADPH consumption the reactions were performed using 1 μM enzyme, 0.2 mM testosterone and 0.1 mM NADPH in potassium phosphate buffer (pH 7.8) at 25 °C; product formation was analysed by HPLC after completion of reaction. *Mutant KSA-2 shows evidences of cooperativity. The Hill equation was used to estimate V_{max} (actually k_{cat} from a plot of v versus S), K_M ($S_{0.5}$ in Hill equation) and the Hill coefficient (n_H). †Coupling efficiency (CE): coupling of product formation to NADPH consumption. ‡Rate of product formation (R). Calculated from the NADPH consumption rates and the percentage of coupling efficiency (see Supplementary Information). §Parameters not given by respective kinetic equations. Mean values and standard errors from triplicates.

with NADPH consumption has been noted in previous cases of protein engineering of P450 enzymes[42].

Investigating the source of evolved regioselectivity. The interpretation of regio- and stereoselectivity in CYP-catalysed C–H hydroxylation is not a trivial task[16–18,21,29,43], especially when complex substrates are involved, as in the present case. To throw some light on the origin of C2- and C15-regioselectivity in the reaction of testosterone (**1**), we performed molecular dynamics (MD) simulations and substrate docking. Mutant KSA-2 from library A, displaying 2β-selectivity, was chosen for the theoretical studies, whereas mutant KSA-14 served as the catalyst for C15-selectivity. To compare these mutants with the catalytic profile of the enzyme used as a parent, P450 BM3(F87A) was included in the MD simulations and docking experiments. MD simulations of the resting state of the enzyme having water-complexed haem-Fe were performed with the Desmond program[44] and OPLS-AA/SPC force fields. Recorded protein conformations were clustered using the Gromos algorithm[45]. For substrate dockings (testosterone, **1**), the Glide program[45] was applied over energy minimized average structures from every cluster of conformers. At this point a decision had to be made as to which Fe-species should be used in docking analyses following the MD simulations. We made use of the QM-derived force fields of Cpd I[46,47] (Fig. 1a), and devised a realistic docking approach by neutralizing the partial positive charge on the active oxygen. This reflects the real steric situation and prevents docking artefacts not consistent with the catalytic mechanism.

The results of studying the parent enzyme P450 BM3(F87A) show that testosterone (**1**), once inside the binding pocket, can exist in essentially only two active positional orientations (poses) as shown schematically in Fig. 2a,b. We define active poses as those in which the distance between the oxygen atom of haem-Fe=O and the carbon atom of the position being oxidized is less than 3.8 Å. The respective O–H–C angle is in the range 160–180°, and the Fe–O–H angle is within 110–140° (refs 40, 48). It can be seen that the two energetically equivalent positional orientations are poised to undergo exclusive attack at the 2β and 15β hydrogen atoms, respectively. This model provides a rationale for understanding the equal distribution of the two regioisomers as well as the source of strict β-diastereoselectivity. To make the alternative α-attack possible, the substrate would have to adopt an active position characterized by 180° reorientation, for which no computational evidence was found.

Analysis of the computational data regarding the 2β- and 15β-selective mutants leads to a very different picture, because the two respective binding pockets now have quite different shapes. The data reveal that the 2β-selective mutant KSA-2 allows for only one active positional orientation of testosterone (**1**), an alignment in which solely the 2β-hydrogen points to the haem-Fe (Fig. 2c). In striking contrast, the computed binding mode in the case of the 15β-selective mutant KSA-14 shows the substrate to be aligned differently, in this case with the 15β-hydrogen in ideal

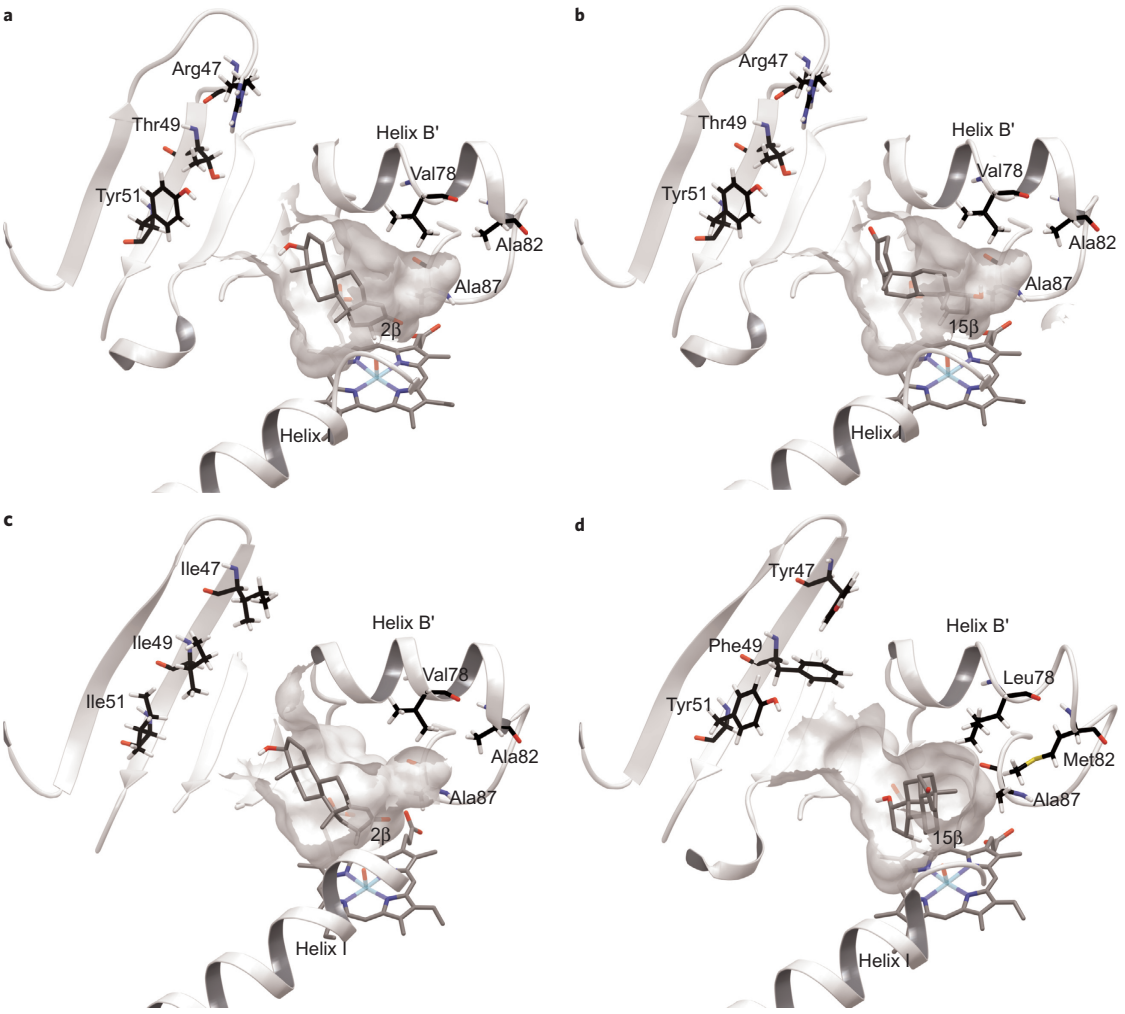


Figure 2 | Computational results of MD simulations and dockings. a,**b**, Parent enzyme P450 BM3 (F87A), demonstrating the two active positional orientations of testosterone (**1**) of equal energy, leading to 2β-hydroxylation (**a**) and 15β-hydroxylation (**b**), respectively, thus explaining 1:1 regio-attack. The active site cavity is partially presented in grey, and amino-acid residues in black. **c**,**d**, The 2β- (KSA-2) and 15β- (KSA-14) selective mutants, each showing the positional fixation of substrate **1** above the haem-Fe for selective 2β-attack (**c**) and 15β-attack (**d**), respectively.

proximity to the haem-Fe (Fig. 2d). Computational evidences for alternative active positional orientations were not found. Indeed, in each case testosterone appears to be 'locked in' due to the complementary topology of the walls of the respective binding pockets. This computational approach was also successful in explaining the results regarding the hydroxylation of progesterone (**4**) (data not shown).

Conclusions and perspectives

This work has shown that one of the current challenges in synthetic organic chemistry, namely the control of regio- and stereoselective oxidative C–H activation of complex organic compounds[3,13,30], can be met by applying knowledge-driven directed evolution to P450 enzymes. We have illustrated this by using the monooxygenase P450 BM3(F87A) as a catalyst in the hydroxylation of testosterone (**1**), which delivers a 1:1 mixture of the respective 2β- and 15β-alcohols. By applying ISM at sites surrounding the binding pocket[20], mutants displaying 96–97% regioselectivity in favour of the 2β- or 15β-isomers, respectively, were evolved on an optional basis without any erosion of diastereoselectivity (β-selectivity). Moreover, the mutational changes induce increased coupling of product formation with NADPH consumption. The efficiency of the ISM approach is not only visible from the actual results, but also from the limited amount of screening, the step that has traditionally been considered the bottleneck of directed evolution[19]. The analysis of MD simulations and substrate docking experiments shed light on the possible source of regioselectivity. The shapes of the binding pockets of the 2β- and 15β-selective mutants have been evolved to enforce in each case a single active positional orientation of the substrate, setting the stage for the respective regio- and diastereoselective oxidation. Further theoretical work is necessary to corroborate this model, which may then serve as a guide in the challenging problem of inverting diastereoselectivity at the 2- and 15-positions.

We conclude by making a recommendation for future practical applications, specifically in view of the fact that more and more P450 enzymes are being discovered and characterized[24,49–52]. If a specific position in a structurally simple or complex organic compound needs to be hydroxylated with complete selectivity, and the screening of known P450 enzymes fails to come up with the desired catalyst, then it is wise to choose one that already mediates at least some oxidation at the desired position. Laboratory evolution as executed in the present study then provides a means to control regio- and stereoselectivity, optionally in combination with chemical tuning based on the use of perfluoro fatty acids as activating additives[53,54].

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Author contributions

S.K. and F.E.Z. performed the experimental work. S.K. and F.E.Z. evaluated the data. J.P.A. carried out the theoretical calculations and analyses. J.P.A. and S.K. performed the kinetics and coupling calculations. M.T.R., S.K. and J.P.A. wrote the manuscript.

Additional information

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