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Engineering CYP153A$_{M.aq}$ to Oxyfunctionalize its Inhibitor Dodecylamine Using a LC/MS Based Rapid Flow Analysis Screening

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The catalytic space of the P450 monooxygenase CYP153A$_{M.aq}$ was opened from a terminal (ω-) fatty acid hydroxylase to a catalyst capable of performing ω-hydroxylation of dodecylamine, which is a potent inhibitor for the wild-type enzyme. A simple screening method named Rapid-flow Analysis of Product Peaks (RAPP) was established and applied to measure saturation libraries directly from a 96-deepwell plate in 36 seconds per sample. The obtained variants are less inhibited by the amine, although concurrently show less affinity towards the acid. Molecular modelling and molecular dynamics simulations showed significant effects of the mutations on the substrate tunnel architectures.

Within the present study, we aimed to oxyfunctionalize a primary alkylamine (generically represented as R–NH$_2$) to the corresponding hydroxylated product (HO–R–NH$_2$). Such amino alcohols can serve as valuable precursors for the production of pharmaceutical substances, like various β-blockers comprising 2-amino alcohols, or biopolymers, such as polyamides, which can be derived from ω-amino alcohols.[1–3] We employed CYP153A$_{M.aq}$ from *Marinobacter aquaeolei* VT8 DSM 11845 as catalyst, which has been part of different studies in our group as a role model for excellent ω-selectivity.[4–6] The co-crystallized model product of CYP153A$_{M.aq}$, ω-hydroxydodecanoic acid, differs from dodecylamine, or its terminally hydroxylated product, only in the functional group, thus the latter was used as a representative for alkylamines. However, dodecylamine acts as an inhibitor for our wild type enzyme catalyst, as do other alkylamines which were previously described as common P450 inhibitors.[7] Typically, enzyme inhibitors are small molecules that interact directly with the enzyme and therefore impair the catalyzed reaction. Inhibitors are often structurally similar to the enzyme's substrate or product, and bind tightly to the active site in the case of competitive inhibition.[8] Depending on the nature of the interactions with the protein, they are basically divided into two types of inhibition: reversible and irreversible, with the latter leading to covalent binding of the inhibitor and thus resulting in the inactivation of the enzyme. However, there are exceptions, such as quasi-irreversible inhibition, in which the inhibitor is not covalently but very strongly bound in the active site.[9]

For cytochrome P450 monooxygenases (P450s), many types of inhibition are well described, including the mechanism-based quasi-irreversible inhibition.[10–12] In this case, the ligand requires initial activation to generate an intermediate that leads to the catalytically inactive metabolic-intermediate complex (MIC), in which the intermediate is tightly coordinated to the ferrous (Fe-II) form of the heme iron.[9,12] Aliphatic and aromatic amines have been described as such quasi-irreversible inhibitors for P450s.[7,11,13] A primary amine is oxyfunctionalized to the corresponding nitroso compound (with the R–N=O functional group), which presumably coordinates tightly to the Fe-II.[12,14] This process can be monitored as a shift in the Soret spectrum from 420 nm to 425–435 nm with the strong-field ligand.[11,15]

Coordination of the amino group to the heme iron would cause the inhibitory MIC, hence we want to circumvent this coordination by anchoring the amino group distantly from the active iron to avoid the formation of the MIC. We have targeted for mutagenesis the residues lining the substrate tunnel that surround the co-crystallized hydroxy dodecanoic acid functional group in the CYP153A$_{M.aq}$ crystal structure (PDB ID 5FYG). We hypothesized that mutagenesis at these positions could enable terminal hydroxylation of dodecylamine. For the engineering by saturation mutagenesis, an efficient and rapid screening method is essential, thus we established a new analysis method based on multiple injections in a single experimental run (MISER chromatography) using flow injection analysis (FIA) without chromatographic separation of the analytes.[16,17]

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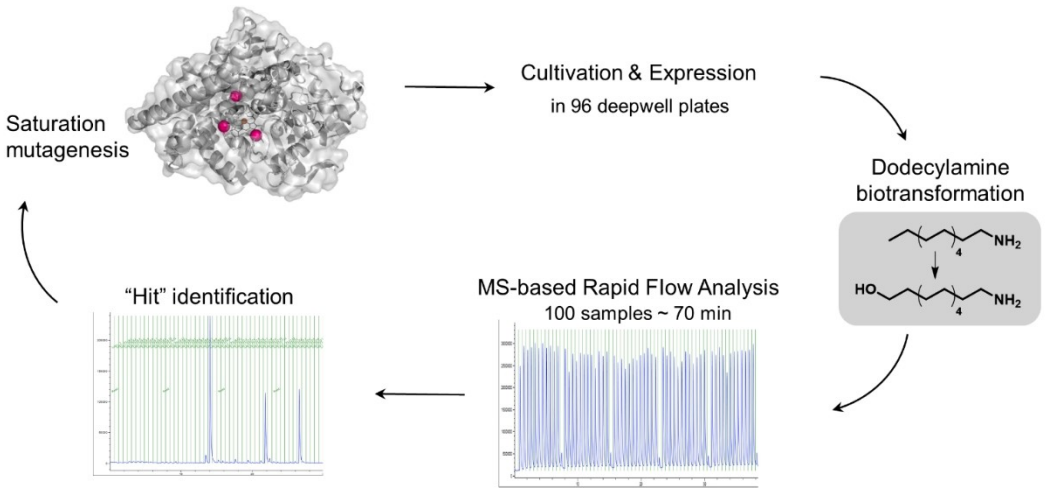

Figure 1. Schematic representation of the Rapid-flow Analysis of Product Peaks (RAPP) screening. A saturation mutagenesis library was cultivated and expressed in 96-deepwell plates. After the dodecylamine biotransformation, the samples were measured by Rapid-flow plates. After the dodecylamine biotransformation, the samples were measured by Rapid-flow identified, evaluated by GC/MS, and available for iterative mutagenesis.

We screened a library of 44 CYP153A$_{M.aq}$ single and double variants with alanine and tryptophane substitutions on residues lining the enzyme tunnels for initial terminal hydroxylation of dodecylamine via GC/FID, and for the first time we identified a double variant V141A/M228A (M.aqAA) hydroxylating trace amounts of dodecylamine. Noteworthy, both amino acids V141 and M228 have previously shown a significant impact on the substrate specificity when combined with Q129, and were therefore selected for saturation mutagenesis.[18]

We established a simple and quick high-throughput screening based on a Rapid-flow Analysis of Product Peaks methodology (RAPP; Figure 1). Within 36 seconds, one sample is injected directly from the 96-deepwell plate (DWP) into an LC/MS system and measured without chromatographic separation of the analytes by omitting a column. The detection was achieved solely by measuring the specific molecular masses of the analytes (SIM-mode; positive ionization; dodecylamine: *m/z* 186; hydroxy-dodecylamine: *m/z* 202). The three positions Q129, V141 and M228 were saturated individually using the 22c-trick.[19] To screen the designed libraries, cells containing the mutated plasmid were cultivated, expressed in DWPs and biotransformations with 1 mM dodecylamine were performed with resting cells. The subsequent extraction and phase separation step allowed the organic phase to be injected directly into the LC/MS system applying RAPP to detect improvements in product formation. Hits were immediately identified by analyzing the resulting single chromatograms (Figure S1–S3). The determined hits were verified regarding the terminal hydroxylation for each round with normalized P450 concentration and GC/FID analysis using the product standard 12-amino-1-dodecanol for quantification (Figure S4). The variant with the highest product formation served as new parent for the next round of saturation mutagenesis. The first single variant Q129E showed an explicit peak in the mass spectrum, but the conversion was too low to quantify (Figure S4). Nevertheless, this variant served as the parent for the next round, since we could not identify any hits from saturation of the other positions (V141 and M228). The consecutive double variant with the highest product formation, Q129E/M228E (M.aqEE), showed 1 turnover per hour, which is 2-fold more than the initial variant M.aqAA. In our study, the third amino acid included, V141, led to the triple variant Q129E/V141S/M228E (M.aqESE), capable of almost 10 turnovers per hour, an 18-fold increase compared to M.aqAA (Figure 2). By introducing two glutamate residues, one of them at the location of a previously described anchoring position for medium chain fatty acids (Q129), we assumed that the amino group of the substrate forms hydrogen bonds to at

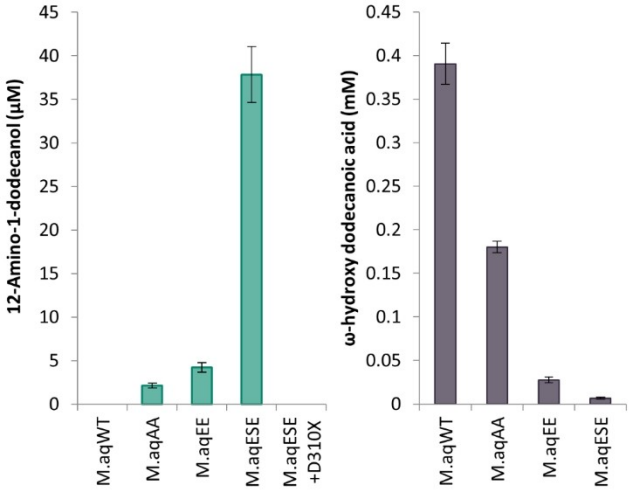

Figure 2. Product formation of the biotransformations using dodecylamine and dodecanoic acid with the wild type CYP153A$_{M.aq}$ and the generated variants. The TON ($n_{product}/n_{P450}$) after 1 h for dodecanoic acid (grey) or 4 h for dodecylamine (green) of conversion for the respective substrates is presented. Error bars result from triplicates.

least one of the introduced carboxylates. This hypothesis was supported by our molecular docking calculations (Figure S5). Accordingly, the alkyl chain reaches towards the heme iron where the terminal carbon would be hydroxylated.[5,18] Further evidence for this coordination hypothesis was given using dodecanoic acid, which serves as a model substrate for M.aqWT with 390 turnovers in one hour (Figure 2, Table S2). Overall, we were able to demonstrate that the ability of the new variant to convert the fatty acid decreases by 58-fold, from 390 to 7 turnovers per hour, while the conversion of the amine increases from 0 to 38 turnovers in 4 h (Figure 2). According to our docking calculations, the catalytically active conformation of this substrate presents the acid group located between the three addressed amino acids, which would be repelled by the glutamate residues (Figure S6). These findings allow explaining why the new variant does not favor the catalysis of dodecanoic acid as much as the wild type, conversely to the trends observed with the dodecylamine.

We further aimed to investigate the modifications of the substrate tunnel architectures to disclose more in depth the molecular basis of the newly-found activity, and highlight their importance in enzyme engineering. We therefore constructed the *in silico* models of the two variants (M.aqAA and M.aqESE) based on the wild type crystal structure (PDB ID 5FYG) and calculated the tunnels using the CAVER PyMOL Plugin.[20] Three different tunnels were identified and assigned according to Cojocaru *et al.* as the substrate tunnels 2c and 2e and a putative solvent tunnel (Figure 3).[21] Neither the Solvent (S) tunnel nor the 2e tunnel were affected by the introduced mutations, whereas tunnel 2c changed dramatically regarding its radius and topology. While it was not surprising to have widened this tunnel by introducing two alanines (M.aqAA), it was more unexpected to have achieved the same effect by the triple mutation M.aqESE. That is, the bottleneck that exists in the wild type at the upper end of the 2c tunnel was fully removed in M.aqAA, while it remained in M.aqESE but with a wider radius (Figure 3). In a recent study, we found that the tunnel 2c was significantly narrowed by three other mutations at the same positions (Q129R/V141L/M228T; M.aqRLT), which produced a new bottleneck directly above the active pocket. This led to a smaller active pocket, reduction in flexibility and, consequently, to a better positioning of the acid substrate in the reactive orientation.[18] Simultaneously, a substrate anchor for fatty acids was introduced by the mutation Q129R. Here we were able to achieve the same kind of H-bridge binding (N–H–O) with the amine group as in that previous work, by using a glutamate instead of a glutamine at the same position (Figure S5).

Using molecular dynamics (MD) simulations of M.aqESE, we found that the mutated residues V141S and M228E can reorient themselves towards the tunnel, and often form a hydrogen bond with one another. Q129E also formed a hydrogen bond with the side chain of S140, which was present in during most of the simulations. Occasionally, V141S can also form a hydrogen bond with the other mutated residue, Q129E (Figure S7). These observations, combined with the docking mode of dodecylamine discussed above (Figure S5), strongly suggest that all three mutations can participate synergistically in an extended hydrogen-bond network with the amine group of that substrate, thus holding it tightly in its reactive mode towards hydroxylation of the terminal carbon. The MDs also

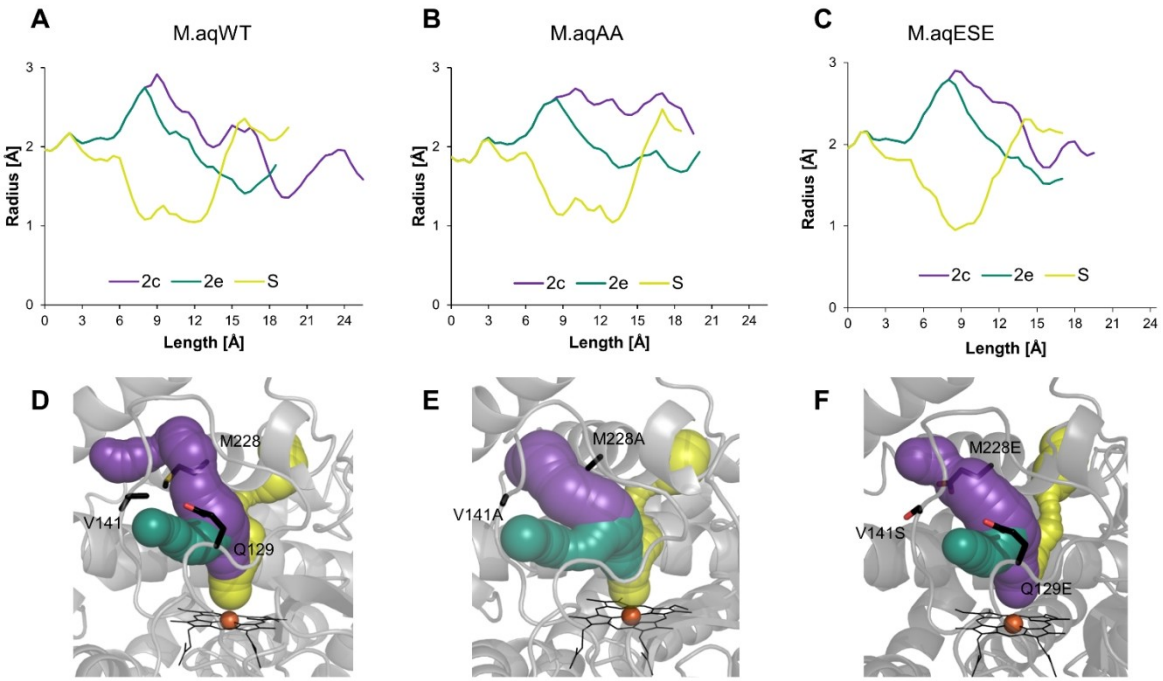

Figure 3. Tunnel profiles and topologies in the structures of the wild type and the investigated variants. Visualization of the 2c (purple), 2e (green) and S (yellow) tunnels in MaqWT (A, D), MaqAA (B, E) and MaqESE (A, D). The heme and mutated residues are represented as black sticks and the iron as orange sphere.

demonstrated that the mutations do not considerably alter the flexibility of the variants compared to the wild type (Figure S8). Exception is for M.aqAA, which seemed to be slightly more flexible in the FG-loop region than the other variants. In contrast, the substrate tunnel 2c is significantly widened and more open in the variants M.aqAA and M.aqESE than for the wild type (Figure S9, Table S3). Such changes seem to arise mostly from a different orientation of the residues around tunnel 2c, mostly due to a decrease in the hydrophobic character and bulkiness of the mutated residues at positions 141 and 228. We therefore can speculate about the possible influence of the higher opening rates observed for the tunnel 2c in reducing the inhibitory effect of dodecylamine in the new variants. We hypothesize that the overall observed effects might be due to a favorable combination of: 1) a higher accessibility of the active site to bind the substrate, due to the higher opening rates of the molecular tunnels (in both M.aqAA and M.aqESE variants), and 2) the anchoring effect of the mutations to hold the amino group in the upper part of the tunnel, thus preventing its binding deeper into the active site and coordinating to the heme (in the M.aqESE variant).

To experimentally assess the coordination of the amino group of the substrate towards the heme iron, we analyzed the spin-states, as these differ depending on whether a substrate (type I) or a strong inhibitor is bound (type II).[15,22] While the Soret maximum is shifted from 420 nm to 385–390 nm for type I spectra, it is displaced to 425–435 nm for type II spectra. We could demonstrate comparable spectral dissociation constants (K_S) for the wild type and the double variants (Figures S10–S13) suggesting similar affinities towards the ligand. Nevertheless, while the binding affinities appear to be similar, a significant difference in the absorbance spectra of the variants was observed. M.aqEE and M.aqESE showed strongly reduced absorbance maxima (Figures S12–13), suggesting that the ligand might rarely coordinate with the ferrous state of the iron Fe(II) and is more likely to be positioned with the amino group in the opposite direction – toward the mutated residues and thereby in a productive mode. These results indicate that the variants M.aqEE and M.aqESE are less inhibited than M.aqAA and the wild type, and are therefore capable of hydroxylating dodecylamine on the other terminal end (Figure 2). Since there would be a shift from type II to type I difference spectra when the terminal carbon is coordinated to the iron instead of the amino group, the curves could partially superimpose between 390–420 nm and thus be distorted, making a clear statement difficult.

To further demonstrate the reduction of inhibition by dodecylamine compared to the wild type, we performed kinetic measurements using dodecanoic acid as the substrate with and without dodecylamine present in the reaction. Since the variant M.aqESE and M.aqEE showed only poor conversion of dodecanoic acid after the evolution towards dodecylamine as substrate, we used M.aqAA and the wild type as representatives instead. Interestingly, we observed considerable substrate inhibition for M.aqAA using dodecanoic acid while the Km was similar to the wild type (Table 1).

Table 1. Kinetic parameters of $CYP153A_{M.aq}$ wild type and the variant M.aqAA for the model substrate dodecanoic acid. Data were collected with and without dodecylamine as inhibitor at the respective IC_{50}.[a]

	M.aqWT	M.aqWT (at IC_{50})	M.aqAA	M.aqAA (at IC_{50})
K_m [mM][b]	0.122	0.66	0.119	0.39
V_{max} [mU][b]	0.079	0.043	0.046	0.064
k_{cat} [min^{-1}]	4.63	0.42	2.72	0.63
k_{cat}/K_m [min^{-1} mM^{-1}]	38.0	0.64	22.8	1.62
IC_{50}[c]	0.9		2.5	

[a] Conditions: 0.1 μM to 0.3 μM P450, 0.08 μM to 1 mM dodecanoic acid, 2% DMSO, 1 mM NADH and cofactor regeneration, 100 mM potassium phosphate buffer, pH 7.4. Concentration ratio used for the reductase partners 1:5:10 CYP153A*M.aq*:CamA:CamB. [b] Derived from Michaelis-Menten equation. cAt 1 μM P450 concentration.

However, when dodecylamine was used as the inhibitor at the IC_{50} (Figure 4 and Table 1), M.aqWT was strongly inhibited (K_m highly increased; v_{max} decreased), whereas M.aqAA was less inhibited at a 2.5-fold higher IC_{50} (K_m increased less than for the wild type; v_{max} increased). It appears that this variant is a less efficient catalyst for dodecanoic acid but is not inhibited as strongly by dodecylamine as the wild type, although the concentration of dodecylamine used was 2.5-fold higher. These data suggest that dodecylamine cannot be assigned to a

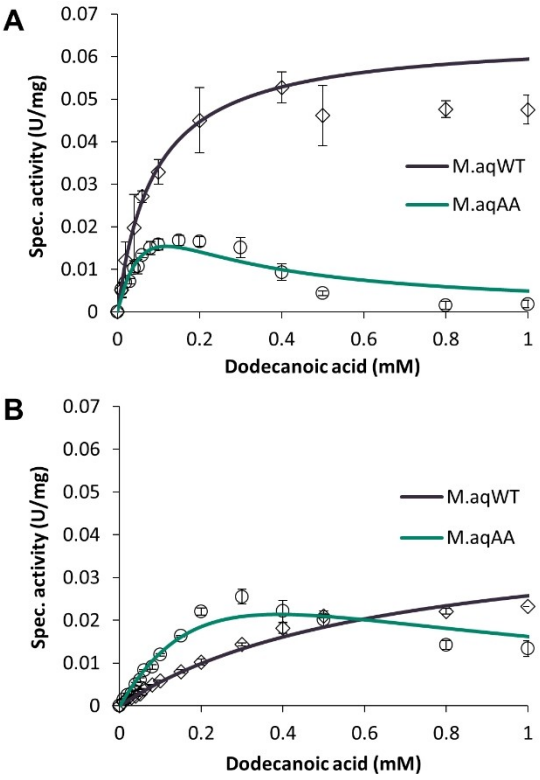

Figure 4. Michaelis-Menten fit to data from kinetic measurements of M.aqWT and the variant M.aqAA using dodecanoic acid as substrate. Data were collected without (A) and with dodecylamine as the inhibitor (B) at the respective IC_{50}. Error bars were calculated from triplicates. The Excel solver plugin was used to fit the data without (M.aqWT) and with substrate inhibition (M.aqAA).

classical inhibitor model, as shown for n-octylamine by Testa and Jenner.[11]

As we have demonstrated in a recent example, the synergy of different factors, namely the substrate anchoring, modification of the tunnel geometry, and changing the flexibility of loops and tunnels, can explain improvements in catalytic efficiency.[18,23,24] Within the scope of this work, we show another example that addresses these factors, but in an extended way: by widening a substrate tunnel, a former inhibitor could be converted into a new substrate. Hence the resulting variant is significantly less inhibited by dodecylamine. Kokkonen and colleagues recently demonstrated the control of substrate inhibition by tunnel modifications in haloalkane dehalogenases.[25] Our presumption of the inhibitory effect relies on the correct orientation of the substrate molecule, whether it acts as an inhibitor or as a possible substrate for the enzyme. With the anchoring of the amino group of dodecylamine in the substrate access tunnel, the alkyl chain reaches towards the catalytically active iron, where the terminal carbon could be hydroxylated. This work has successfully demonstrated the importance of considering engineering positions beyond the active site to convert molecules that were previously not catalyzed or even inhibited the reaction. The resulting M.aqESE variant could serve as a parent for further rounds of mutagenesis to a highly efficient hydroxylase for aliphatic amines with suppressed inhibition by the substrate. This engineered enzyme opens the catalytic space of this enzyme subfamily to a new group of molecules.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: biocatalysis · cytochrome P450 · docking · enzyme engineering · molecular dynamics

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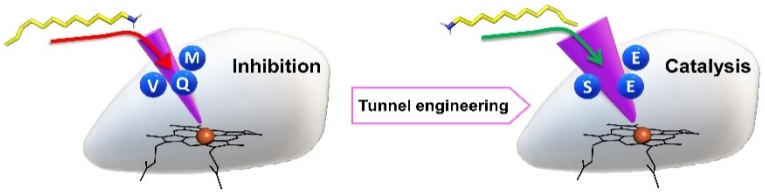

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A light at the end of the tunnel: A P450 inhibitor, dodecylamine, was accepted as substrate after iterative site saturation mutagenesis on three opposing tunnel lining residues. The substituted amino acids changed the tunnel architecture and furthermore prevent the amino group getting too close to the catalytic iron resulting in inhibition. A MS-based rapid analysis was used as pre-screening to find variants capable of catalyzing the hydroxylation of the former inhibitor.