

Substrate Anchoring and Flexibility Reduction in CYP153A_{M.aq} Leads to Highly Improved Efficiency toward Octanoic Acid

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ABSTRACT: Cytochrome P450 CYP153A_{*M.aq*} from *Marinobacter* aquaeolei serves as a model enzyme for the terminal (ω -) hydroxylation of medium- to long-chain fatty acids. We have engineered this enzyme using different mutagenesis approaches based on structure-sequence-alignments within the 3DM database and crystal structures of CYP153A_{*M.aq*} and a homologue CYP153A_{*P.sp*}. Applying these focused mutagenesis strategies and site-directed saturation mutagenesis, we created a variant that ω -hydroxylates octanoic acid. The M.aqRLT variant exhibited 151-fold improved catalytic efficiency and showed strongly improved substrate binding (25-fold reduced K_m compared to the wild type). We then used molecular dynamics simulations to gain deeper insights into the dynamics of the protein. We found the tunnel modifications and the



two loop regions showing greatly reduced flexibility in the engineered variant were the main features responsible for stabilizing the enzyme-substrate complex and enhancing the catalytic efficiency. Additionally, we showed that a previously known fatty acid anchor (Q129R) interacts significantly with the ligand to hold it in the reactive position, thereby boosting the activity of the variant M.aqRLT toward octanoic acid. The study demonstrates the significant effects of both substrate stabilization and the impact of enzyme flexibility on catalytic efficiency. These results could guide the future engineering of enzymes with deeply buried active sites to increase or even establish activities toward yet unknown types of substrates.

KEYWORDS: biocatalysis, enzyme engineering, molecular dynamics, computational chemistry, cytochrome P450

INTRODUCTION

Nowadays, enzymes are recognized as highly dynamic molecules and no longer as static structures. Targeting the flexible parts of enzymes has often been shown to influence the dominant conformations of the protein, which sometimes can be advantageous for more efficient catalysis.^{1,2} However, for simplification, we still often work with the static crystal structures of proteins for (semi-) rational enzyme engineering. In contrast, addressing highly flexible structural elements such as loop regions and molecular tunnels, rather than the active site, has gained more and more interest in biocatalysis.^{3–5} The potential of tunnel engineering to improve or achieve (new) enzymatic functions is acknowledged but is experimentally still very underexploited, although many predictive simulations and retrospective elucidations exist.⁶⁻⁸ Nevertheless, Ebert and colleagues demonstrated the successful prediction of an unknown substrate-specific hotspot for activity by using biased molecular dynamics simulations where they evaluated the substrate's pathway into the active site.9 This path or empty void that guides molecules between the catalytic site and the surrounding solvent is referred to as a molecular tunnel. While more than 64% of enzymes contain tunnels longer than 15 Å

leading to the buried active site, the majority of enzymes with such long tunnels are oxidoreductases.¹⁰ Among these, cytochrome P450 monooxygenases (P450s or CYPs) represent an example for well-studied tunnel occurrence and architecture.¹¹

P450s are a heme-iron containing superfamily of more than 52 000 known sequences spread among all the kingdoms of life (CYPED database https://cyped.biocatnet.de/sequence-browser).¹² These enzymes are known to catalyze the chemically fundamental oxidation of nonactivated carbon atoms in a highly selective manner. The regioselective oxidation of the unactivated terminal carbon of alkanes and fatty acids compared with other positions is a major challenge especially in chemistry and also P450 engineering. Chemically, the terminal oxidation to hydroxycarboxylic acids can be

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Figure 1. Mutagenesis scheme, terminal hydroxylation of octanoic acid, and product formation with wild type CYP153A_{*M.aq*} (PDB ID 5FYG) and designed variants. Three single positions were mutated after a consensus alignment within the 3DM database (red), comparison with the homologue CYP153A_{*P.p*}(PDB ID 7AO7) (blue) or substituted with amino acids found in a saturation mutagenesis approach (gray). The best hits were combined (green) and led to the best variant RLT (QR/VL/MT) known so far. The formation of ω -hydroxy octanoic acid (C8-FAOH) is shown for each variant. The dashed line indicates wild type product formation. Conditions: 1 μ M P450, 5 μ M CamA, 10 μ M CamB, 1 mM octanoic acid, 2% DMSO, 1 mM NADH and cofactor regeneration, 100 mM potassium phosphate buffer, pH 7.4, 550 rpm, 30 °C, 1 h.

achieved by the selective oxidation of aliphatic diols or the hydrogenation of dicarboxylic acids but not by selectively hydroxylating the corresponding fatty acid, because of the strong primary hydrocarbon (C–H) bond (>100 kcal/mol).^{13–16} Whereas numerous subfamilies of P450s catalyze the regioselective oxyfunctionalization of fatty acids to hydroxy fatty acids at subterminal positions, the terminal (ω -) position is hydroxylated mainly in C12 and longer saturated fatty acid substrates.¹⁷ In our group, we have previously obtained a ω -hydroxylase variant of CYP153A_{M.aq} from Marinobacter aquaeolei that can selectively catalyze the terminal hydroxylation of C9–C20 fatty acids.¹⁸

Among the many sources to find efficient enzymes for desired chemical reactions (natural diversity, genome mining, directed evolution, and rational design), in today's third wave of biocatalysis more engineering and computational approaches are applied than the direct use of the extensive naturally available resources.¹⁹ The group of Frances Arnold, for example, applied five rounds of directed evolution on the medium-chain (C12 to C18) fatty acid hydroxylase P450 BM-3, leading to an alkane hydroxylase that is able to convert octane.²⁰ Out of 11 random mutations, at least five were retrospectively identified as binding pocket and tunnel lining residues, while the others were randomly distributed over the entire heme domain. In order to find new enzymes, extensive database research, metagenome libraries, and enzyme mining are the technologies available today. Although purely computational designs rarely present outstanding results, they are often combined with directed evolution methods in protein engineering, sometimes with extraordinary results.²¹⁻²³ However, for the highly efficient approach of directed evolution,

suitable screening systems are usually the bottleneck that are absolutely necessary.

In this work, we aimed to get a deeper insight into the dynamics of an enzyme when it is evolved toward accepting a smaller substrate. We chose $CYP153A_{M.aq}$ because of its excellent ω -selectivity for medium- to long chain fatty acids (C9-C20) and chose to engineer it toward the conversion of smaller substrates (C8). We applied three different engineering approaches, which mainly had moderate effects on the product formation but eventually generated highly improved variants by simply combining the best mutations (Figure 1). The wild type (WT) and the best catalyst were then computationally analyzed and the results were evaluated to gain deeper insights into the structure-function relationships. With the mutation Q129R, which was previously described as C12 fatty acid anchor, we created an anchor to also hold the smaller substrate (C8) in place, while the other mutations possibly reshaped the tunnel and reduced the flexibility for more efficient catalysis. Furthermore, we showed that the ligand is substantially more stabilized in the active position in the mutant than in the wild type. We thereby created a variant with a shift in substrate specificity with respect to the preferred carbon chain length compared with the wild type. The resulting knowledge about the influence of tunnel lining residues and how the protein dynamics can potentially be addressed could serve as a basis for future enzyme engineering strategies.

RESULTS AND DISCUSSION

Combination of Hits from Different Mutagenesis Approaches Leads to Strongly Improved Hydroxylation. In order to extend the catalytic spectrum of CYP153A_{Mag} toward shorter substrates than previously described, we substituted tunnel lining residues distant from the catalytic site.¹⁸ Our group has already demonstrated the de novo production of $\hat{\omega}$ -hydroxyoctanoic acid in whole cells by coexpressing a thioesterase to overproduce octanoic acid from the natural fatty acid cycle and an artificial fusion construct CYP153A_{M.aa}(G307A)-CPR_{BM3} for the corresponding ω hydroxylation.²⁴ The mutation G307A is located in the active site near the heme, which was not our target. Additionally, the whole cell approach was performed for 20 h in a fed-batch reactor, compared with our new 1 h small-scale screening system with lysate and free reductases with excellent reproducibility. Thus, we wanted to gain more mechanistic and dynamic insights than previously achieved. In our preliminary work, we observed three residues positioned in a horizontal plane around the model product ω -hydroxy dodecanoic acid in the structure of wild type CYP153A_{*M.aq*} (PDB ID 5FYG).²⁵ These residues form one section of the molecular tunnel 2c, which was identified to be the favored substrate entrance tunnel (CaverDock, Figure S4 and Table S5). The first position, Q129, has already been described by our lab as a fatty acid anchor for dodecanoic acid (C12) when mutated to an arginine (Figure 2).²⁵ The second position, M228, confers an altered specificity toward fatty acid chain lengths depending on the amino acid substitution (data not shown). Finally, we focused on V141 as the third amino acid of this triangular relationship and hypothesized that these residues would influence each other.

Here, we used different mutagenesis approaches to improve the CYP153A_{*M.ag*} activity toward octanoic acid: (i) A structuresequence-alignment in the 3DM database (https://3dm.bioprodict.com/) of 312 P450 enzymes with a sequence identity of \geq 55% compared with CYP153A_{Mag}; (ii) A homologous enzyme CYP153A_{P,sp} with a sequence identity of 56% and known to hydroxylate the shorter substrate octane was crystallized, and its structure was determined in complex with the hydroxylation product octan-1-ol. These structures of CYP153_{*M.aq*} and CYP153A_{*P.sp*} were aligned, and the corresponding amino acids were substituted; (iii) The target positions were individually saturated with the 22c-trick²⁶ and prescreened with a whole-cell screening using an artificial fusion construct to lower the variation particularly established for fatty acid hydroxylation; (iv) Finally, the best single mutations were combined to create double and triple variants. The triple mutant with the best activity toward octanoic acid, M.aqRLT, had the mutations Q129R, V141L, and M228T.

After 1 h, wild type $CYP153A_{M.aq}$ showed the conversion of 1.8% octanoic acid to ω -hydroxy octanoic acid. The structuresequence-alignment in the 3DM database (Figure 1, red; Figure S2) revealed conserved amino acids at two of the positions. These were subsequently substituted to V141I and M228L. It is noteworthy that the position Q129 did not have a consensus number in the 3DM database and was therefore not considered. The substitutions of the corresponding residues from CYP153A_{P.sp} were Q129N, V141L, and M228A, respectively (Figure 1, blue). The single variant M.aqV141L showed the highest improvement of $117.9 \pm 16.4 \,\mu\text{M}$ product formation, a 7-fold increase compared to the wild type. The individual saturation of the three positions (Figure 1, gray) led to the previously known fatty acid anchoring residue Q129R and further to V141M and M228T. The variants were evaluated under normalized conditions, showing approximately 3-fold improved product formation compared to the wild type.



Figure 2. (A) Representation of the mutated residues Q129R, V141L, and M228T (green) of the best variant M.aqRLT with docked octanoic acid (yellow) and the crystal structure of the wild type residues (gray) with the cocrystallized ω -hydroxy dodecanoic acid (dark green) (PDB ID 5FYG). (B) M.aqWT and M.aqRLT with docked octanoic acid. The distances between the terminal carbon and the iron are represented. The heme is represented as black lines and the iron as an orange sphere.

A combination leading to the double variant M.aqRT (Q129R/M228T) strongly increased the product formation to 10-fold compared to the wild type. Remarkably, the triple variant Q129R/V141M/M228T showed a decrease in the product formation compared to the double variant (Table S4). The mutation V141L was therefore combined with M.aqRT, resulting in M.aqRLT (Q129R/V141L/M228T), the best variant to date for the production of ω -hydroxy octanoic acid with a 14-fold improvement compared to the wild type.

We determined the kinetics to further characterize the wild type and the best variants (M.aqR (Q129R), M.aqRT and M.aqRLT) to show the progress in substrate affinity and the improvement of their catalytic efficiency. For all variants the turnover number, k_{catr} was highly increased, but for M.aqR and M.aqRT it was 1.5 and 1.7 times higher than for M.aqRLT (Table 1, Figure S3). For the wild type, K_m is presented as apparent because the calculated value is above the solubility limit of octanoic acid. As expected, K_m was reduced significantly from the wild type (7.5 mM) to the single (4.1 mM), the double (0.68 mM), and the triple (0.3 mM) variant. Thus, the catalytic efficiency for M.aqRLT proved to be the

Table 1. Kinetic Parameters of CYP153A_{M.aq} Wild Type and Variants R, RT, and RLT for Octanoic Acid^a

	$\binom{K_{\rm m}}{({\rm mM})^{b}}$	$(\min^{-1})^{b}$	$k_{ m cat}/K_{ m m}$ (min ⁻¹ mM ⁻¹)	efficiency improvement
WT	7.5	0.14	0.018	
R	4.1	1.25	0.31	17-fold
RT	0.68	1.38	2.02	112-fold
RLT	0.3	0.81	2.72	151-fold

^{*a*}Conditions: 0.5 μ M to 1 μ M P450, 0.01 mM to 5 mM octanoic 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.

highest with a 151-fold improvement. Because of the significant decrease of the $K_{\rm m}$ for M.aqRLT, we selected this enzyme variant for further computational analyses and comparison with the wild type.

Modeling of M.aqRLT to Understand Its Improved Activity. The variant M.aqRLT was constructed based on the wild type crystal structure (PDB ID 5FYG),²⁵ with the corresponding mutations introduced using Swiss-Model. We performed the molecular docking of the octanoic acid (Figure 2, yellow), and it showed the carboxyl group strongly interacting with the mutated arginine 129. This position had previously been shown to be the carboxyl anchor for longer fatty acids such as dodecanoic acid (C12).²⁵ The acid group of dodecanoic acid is located slightly above the triangular plane in the wild type (Q129, V141, and M228) (Figure 2A). In contrast, the carboxyl group of the octanoic acid (C8) is located slightly beneath the substituted amino acids (Q129R, V141L, and M228T), which delineate the 2c tunnel, far from the active site.

Additionally, we docked octanoic acid into the wild type crystal structure and observed important differences compared to M.aqRLT (Figure 2B). In both enzymes, the carboxyl group points in the direction of the three addressed amino acids, but in the wild type, the terminal carbon (which we have experimentally shown to be the only position to be hydroxylated) is 2 Å more distant from the catalytic iron.



Figure 3. Tunnel architecture of the enzyme variants and protein dynamics accessed by B-factors calculated using molecular dynamics. The BC-loop (pink) and the FG-loop (blue) are highlighted in the structure of M.aqWT (A) and M.aqRLT (B) shown with the substrate tunnels 2c (purple) and 2e (green) and the Solvent tunnel S (yellow). The heme is represented in black sticks and the iron as an orange sphere. (C) B-factors of the enzymes residues with the octanoic acid ligand (oct). M.aqWT + oct (black), M.aqRLT + oct (green) and the enzyme M.aqWT (gray dotted) and M.aqRLT without ligand (green dashed). B-factors were calculated as average of the four individual MD simulations, respectively. The position of the mutations is indicated by the green arrows. The tunnels were calculated using the sofware CAVER 3.2.

The different docking solutions were also more dispersed with the wild type than with the M.aqRLT variant. Because of the deeper positioning of the substrate terminal carbon and thus closer proximity to the heme iron, we hypothesize a more efficient catalysis in the variant. These results provided the first hints of why we have created a better catalyst: the introduced mutations, especially Q129R, seem to be better at anchoring the octanoic acid in the catalytically active conformation to be terminally hydroxylated. This indication was then investigated further with greater computational effort.

Modification of Molecular Tunnels Contributes to Enzyme Activity. The tunnel calculation allows us to compare the enzyme tunnels in the wild type and in the designed mutant variant with respect to their geometric properties and topologies. Compared to the wild type (Figure 3A), the 2c tunnel of M.aqRLT shows a new sharp bend closer to the active site, which is created by the side chain of M228T (Figure 3B). This fact supports the assumption of the substrate being better stabilized in the active site, thus promoting the chemical step. This is also indicated by the lower $K_{\rm m}$ of the variant, which was also demonstrated by Khersonky and colleagues.²⁷ We show that a new bottleneck in tunnel 2c was created by the mutations in variant M.agRLT. Fasan and coworkers similarly illustrated a newly introduced bottleneck after the evolution of BM-3 from a long-chain fatty acid hydroxylase to a propane monooxygenase, in which even the active site was separated from the substrate tunnel.²⁸ In addition, narrow tunnels are described as sterically constrained access pathways to address the terminal position for functionalization in P450s.¹⁶ Since tunnel 2e, with the mutation Q129R at its entrance, is the energetically favored pathway for octanoic acid in M.aqRLT (CaverDock, Figure S4 and Table S5), this supports the hypothesis of a better stabilization of the substrate in the active site or prevention of its egress. We hypothesize that the limiting step for converting octanoic acid is not the migration of the substrate into the active site because the model substrates of the wild type enzyme are also fatty acids, albeit with longer carbon chains (C9-C20), but rather its positioning and stabilization in the active site pocket.¹⁸ This is supported by coupling data; whereas the wild type displayed poor coupling of only 10.5 \pm 1.5%, the variant M.aqRLT showed a 2-fold improved coupling of $19.6 \pm 0.5\%$.

Reduced Flexibility Enables More Efficient Catalysis. To assess the protein flexibility and its influence in the function, we performed molecular dynamics (MD) simulations and analyzed the respective differences with and without octanoic acid bound in the active sites. There are two major regions in which flexibility (assessed by the B-factors) differs significantly between the investigated enzyme variants. These elements correspond to two loops, which partly form the binding pocket, but mainly serve as guiding elements for the substrate tunnels of the enzymes. More precisely, they are referred to as BC-loop and FG-loop, which connect the respective helices (Figure 3A,B). The B-factors of each residue from M.aq wild type and M.aqRLT with (+ oct) and without the octanoic acid were computed for the backbone atoms (Figure 3C). The two main substrate tunnels (2c and 2e) are basically defined by the BC-loop and the FG-loop/G-helix (Figure 3A,B), which are highlighted in Figure 3C. Our results showed that the BC-loop is highly flexible in M.aqWT, and even more in the presence of octanoic acid. M.aqRLT, conversely, displayed B-factors in that region that were

considerably lower (by more than 30%), suggesting increased stabilization and stronger interactions with the bound substrate. Interestingly, we found more than 2-fold higher B-factors in the FG-loop of the variant M.aqRLT compared to the wild type. This difference, due to the introduced mutations, was observed in the free enzyme but not in the presence of the ligand (Figure 3C and Table S6). This may suggest that the access tunnels lined by this loop are more adaptable—thus facilitating the entrance of the substrate—but remain stable after the substrate is bound.

At this position, another tunnel termed 2ac (Figure S5 and Table S6) could emerge in the variant M.aqRLT, which is also known to be present in the crystal structure of the alkane hydroxylase CYP153 $A_{P.sp}$ (P.sp) (Figure S5) which favors shorter chain lengths than CYP153 $A_{M.aq}$.²⁹ Because the variant M.aqRLT is a more efficient biocatalyst for the short octanoic acid (151-fold improved k_{cat}/K_m) compared to the wild type, the opening of a 2ac tunnel could be an advantage for the conversion of this shorter fatty acid. This hypothesis was supported by the analysis of tunnels using CAVER, in which we saw a slight increase in the opening rates of the said 2ac tunnel in M.aqRLT. Nevertheless, it showed a nearly similar presence during the MD simulations of both wild type and mutant. Therefore one can only speculate about a potential role in the enzyme activity. We would assume that the increased flexibility correlates with the tunnel availability, but this is not necessarily the case. We observed a higher presence of the 2c tunnel in M.aqRLT + oct (18% open) compared with M.aq wild type + oct (4.4% open), while the wild type has 1.6fold higher B-factors at the BC-loop (Table S6). Since the 2c and 2ac tunnels are bordered by the BC- and FG-loops (and beginning of the G-helix), these tunnels depend on the movement of both loops as counterparts, and no explicit correlation can be observed. However, the increased flexibility of the FG-loop in the unbound M.aqRLT did induce higher opening rates of the 2c tunnel compared with the wild type (19% vs 1.1%, respectively). As mentioned above, this may have an impact on the rates of substrate binding.

Unexpectedly, we observed that the presence of octanoic acid did not dramatically increase the tunnel bottleneck radii in either of the enzymes compared with the respective unbound systems (Table S6), as observed in other reported cases.³⁰ We found that the narrowest parts of both 2e and 2c tunnels are most often located closer to the tunnel mouth, where the ligand rarely reached during these MDs.

Stabilization of the Ligand in the Active Site. The MDs of the enzymes bound with substrate contained the octanoic acid initially located in the binding pocket, where the tunnels 2c and 2e are merged, and it did not exit the enzyme during any of the four 200 ns simulations. In the variant M.aqRLT, the octanoic acid remained closer to the heme, whereas in the wild type, it moved toward the mouths of both the 2c and 2e tunnels. To track the location of the octanoic acid during the MDs, the histogram (Figure 4A) shows the distribution of the distance between the terminal carbon, which will be hydroxylated during the catalysis, and the catalytically active iron. In the variant M.aqRLT, the octanoic acid showed a single maximum for distances around 4-5 Å from the iron, containing 70% of the MD frames. In the wild type, it presented two maxima and a more widely distributed position along the tunnels. The first maximum corresponds to similar distances as in the variant M.aqRLT, but only for 35% of the frames, and the additional maximum was observed 30%



Figure 4. (A) Histogram of the octanoic acid position, showing the distribution of distances between the terminal carbon of the ligand and the catalytically active iron. It compiles all molecular dynamics simulations with M.aqWT (gray) and M.aqRLT (green). (B) Interaction energy $\Delta G_{\rm bind}$ of the octanoic acid with M.aqWT and M.aqRLT, averaged over all MDs. With the exception of L141, only residues with $\Delta G \geq 0.5$ kcal/mol are displayed. The error bars represent the respective standard deviations from the mean values.

of the time, where the carbon is 11–13 Å distant from the iron. Therefore, the ligand was in a nonreactive position in most of the simulations of M.aq wild type. These results indicate the substrate being better stabilized inside the active pocket of the variant M.aqRLT than in the wild type, which translate in increased probabilities of achieving a reactive conformation.

Previously Known Anchor for Stabilizing Acid **Groups.** The free binding energy ΔG_{bind} of the octanoic acid with the proteins was calculated using the MM/GBSA method and averaged over the four replicated MD simulations, for M.aq wild type and M.aqRLT. ΔG_{bind} was dissected by residue, the averages compiled, and the most interacting residues are presented in Figure 4B. There are two major interesting differences between M.aq wild type and M.aqRLT, which may explain the more efficient catalysis by the mutant variant. In M.aqRLT, ΔG_{bind} of the substrate and the heme iron (HEM) was twice as high as in the wild type (ΔG_{bind} of -2.12 and -1.04 kcal/mol, respectively), indicating a greater interaction with the catalytic site. However, the most striking difference in the ligand interactions was at position 129, which was mutated in M.aqRLT. While ΔG_{bind} was -2.8 kcal/mol for Q129 in M.aq wild type, the mutation to an arginine in the variant M.aqRLT (Q129R) altered ΔG_{bind} to -7.3 kcal/mol, which is a remarkable change. This mutation was previously described as an anchor for longer fatty acids like dodecanoic acid,²⁵ and it may be responsible for keeping the octanoic acid in the reactive position until the reaction is accomplished. As in this study, also in other P450s capable of fatty acid hydroxylation, arginines are commonly described as possible binding sites for the carboxylate.¹⁶ Interestingly, the mutation M228T shows a slight, even reduced, difference compared with the wild type, and ΔG_{bind} for V141L was only -0.11 kcal/mol. We speculate that these two residues scarcely interact with the ligand in the bound conformation, but they may be rather important for reducing the flexibility of the BC-loop and stabilizing the whole construct. Of high relevance could also be the reshaping of tunnel 2c (Figure 3C, purple tunnel), by

which the active site pocket has shrunk considerably, while the arginine at position 129 protrudes and stabilizes the acid group of octanoic acid.

New Substrate Specificity of M.aqRLT. To further demonstrate the suspected difference in the fatty acid substrate specificity between M.aq wild type and M.aqRLT, we investigated the conversion of saturated fatty acids with chain lengths between C6 (hexanoic acid) and C16 (hexadecanoic acid). To determine the turnover number (TON), we used 1 mM of the substrates and the same reaction conditions for all enzymes for comparison.

With the mutant variants we generated generally improved catalysts for the terminal hydroxylation of medium-chain fatty acids. This fact was not unexpected due to the mutation Q129R, which had previously proved beneficial for C12 conversion. The highest product formation was shown with the substrate nonanoic acid (C9) and a slight decrease when using decanoic acid (C10) and dodecanoic acid (C12). The improvement of M.aqRLT relative to the wild type decreased more strongly from 10-fold (C9) to 4-fold (C10) and 2-fold (C12) and was only 62% for the long-chain fatty acid C16 (Figure 5 and Table S7). It is worth mentioning that we see



Figure 5. Fatty acid specificity of the wild type M.aqWT (dark gray) and the variants M.aqR (light gray), M.aqRT (white), and M.aqRLT (green). Substrates were tested regarding their chain lengths between C6 and C16. The TON ($n_{\text{product}}/n_{\text{P450}}$ after 1 h conversion) for every substrate is presented.

the highest improvement in product formation for the octanoic acid (C8) with 14-fold improvement compared to the wild type. We even observed 35 μ M ω -hydroxy heptanoic acid after 1 h reaction time with the variant M.aqRLT and the corresponding C7 substrate, while the wild type showed no product formation under these conditions. For the shortest fatty acid (C6) used in this study, no product formation could be detected. Generally, the variant M.aqRLT demonstrated higher product formation with C8 to C12 compared with all other variants used in this study, demonstrating the importance of not only the anchoring but also the two mutations influencing the flexibility. Fasan and colleagues could even demonstrate the evolution toward a C3 alkane hydroxylase showing similar substrate tendencies regarding the chain length. $^{\rm 28}$ For better stabilization of shorter substrates, amino acids in the tunnel closer to the active site could be targeted using the same mutagenesis approaches. Anchoring mutations were previously introduced more deeply into the tunnel of

BM-3 by groups of Urlacher and Munro to rationally form hydrogen bonds between Arg/Tyr residues and the carboxylate of shorter fatty acids.^{31,32}

CONCLUSIONS

The fundamentals in improving the activity of the CYP153A_{*M.aq*} variant M.aqRLT toward octanoic acid have been elucidated, and it could be shown that slight modifications in the molecular tunnels can lead to altered activities. We have shown that the flexibility of the main loops and the corresponding tunnels is of high importance and that these elements can be used as possible targets for engineering enzyme activity.

A synergy of different factors, such as substrate anchoring in the active site, reshaping of the active site by changing tunnel geometry and reduction of the enzyme's flexibility have been assessed and can explain the strongly improved catalytic efficiency of the designed variant. A deeper and tighter positioning of the substrate inside the active site was demonstrated by the MD simulations, as was earlier shown for Kemp eliminases.²⁷ The mutation Q129R places the octanoic acid in the correct orientation until the hydroxylation reaction is completed. It is known that the catalytic efficacy of enzymes depends on the exact positioning and the suitable electrostatic environment in the active pocket.³³ The other evidence presented in this study suggests that the flexibility of two loops forming the possible substrate access and egress tunnels are substantially reduced compared to the wild type. Therefore, the substrate is better stabilized in the Michaelis complex, and the terminal carbon interacts more frequently with the catalytically active heme.

With deeper insights into the dynamic properties of an enzyme, we might be able to identify and target activity hotspots more specifically and predictably in the future. We could aim for higher activity or broader substrate specificity, even including new molecules that were not previously accepted as substrates.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.0c05193.

Information on crystallographic data collection, detailed experimental and computational procedures, and further supporting figures and data (PDF)

X-ray data for 7ANT (CIF)

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X-ray data for 7ANT (CIF)
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X-ray data for 7AO7 (CIF)

X-ray data for 7AO7 (CIF)

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Author Contributions

L.R.R. designed and performed the experiments, the molecular modeling calculation and analysis and wrote the manuscript. S.M.M., J.D., and L.R.R. designed the molecular modeling studies, S.M.M. supervised the execution and interpretation of the computational results. E.Z., B.R., and M.S. crystallized the protein and determined the crystal structure with G.G. All authors have given approval to the final version of the manuscript.

Notes

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