Structure guided redesign of CYP153A*M.aq* for improved terminal hydroxylation of fatty acids

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**Abstract:** The structure of a P450 ω‑hydroxylase bound to its fatty acid product was determined, revealing a narrow substrate tunnel that leads to the heme. The introduction of an arginine side chain in proximity to the carboxyl group of the fatty acid led to a reduced KM value for dodecanoic acid, suggesting the importance of an anchoring point in the active site. An increase in flexibility of the substrate recognition region was also engineered, resulting in 3-fold improved product formation.

Introduction

The regio- and stereoselective hydroxylation of non-activated C‑H bonds remains a challenge in chemical synthesis.[1] The few reactions that have been described suffer from low selectivity or harsh conditions.[2, 3] Terminally hydroxylated fatty acids (ω‑OHFA) are of great interest in the chemical industry due to their versatile application as building blocks for musk fragrances and biopolymers.[4, 5] In nature this reaction can be catalysed by cytochrome P450 monooxygenases (CYPs).[6] These enzymes are present in all kingdoms of life. Microbial P450 enzymes are of special interest for industrial application because of their ease of handling due to their non-membrane bound form.[7] Amongst the bacterial P450 enzymes several have been described as fatty acid hydroxylases.[8-10] The P450 monooxygenase from *Bacillus megaterium* (P450BM3) is so far known as the most active P450 for the hydroxylation of sp3‑carbons.[11] Since P450BM3 catalyzes predominantly the thermodynamically favoured subterminal hydroxylation several rounds of engineering have been performed in an effort to change its specificity.[10, 12-14] This way the substrate scope[15] and novel activities have been developed.[16] Engineering of the regioselectivity towards the terminal position of fatty acids however, resulted only in enhanced selectivity. For example, Brühlmann and co-workers described an optimised P450BM3 variant with eleven mutations which showed a terminal selectivity of 74 % for palmitic acid.[17]

Terminal hydroxylases, such as CYP153A from *Marinobacter aquaeolei* (CYP153A*M.aq*), have been shown to hydroxylate fatty acids with a chain length from C9 to C20 with a high regioselectivity for the terminal position (> 95 %).[18] This enzyme has been extensively studied in our laboratory and fusion constructs as well as assay systems have been developed.[19-21] In previous studies, a position crucial for the terminal selectivity was determined (L354).[18] The structural reasons for the selectivity, however, have yet to be determined. In a recent approach[22] two different functional protein sites have been studied in a semi-rational mutagenesis based on a homology model. The positions which showed the most pronounced effect on enzyme activity in that study were predicted to be located at the binding pocket close to the heme (G307) and at the substrate entrance (S233). Interestingly, another mutant (T302I) showed an altered substrate specificity in which activity was increased towards medium chain fatty acids (C8, C12), and the conversions for palmitic acid were reduced (C16). In order to explain the activity and selectivity of CYP153A*M.aq,* three major issues need to be addressed:

i) the substrate recognition, ii) substrate anchoring to an amino acid in the active site and iii) the structural basis for the enzyme’s regioselectivity.

Two of these questions have been addressed amongst other using P450BM3 and P450pyr (CYP153A7 from *Sphingomonas* sp. HXN-200). Noble and co-workers studied the P450BM3 residues that are involved in the interaction of the fatty acid carboxylate with the active site of P450BM3. By designing mutants without a polar or charged functional site group (R47A, R47G and Y51F) and then performing kinetic studies of the mutants, the side chains R47 and Y51 were identified as being involved in the recognition of the fatty acid carboxylate.[23] Altering these positions resulted in lower *K*M and *k*cat/*K*M values suggesting the requirement of an efficient recognition point for anchoring the substrate.

In contrast to P450BM3, P450pyr – which belongs to the CYP153A family – shows a pronounced terminal selectivity compared to P450BM3. The functional sites were engineered to give mutants that catalysed subterminal hydroxylations by enlarging the binding pocket at the heme-distal site.[24] The extended pocket permitted a horizontal orientation of the substrate in relation to the heme which led to a preference for subterminal hydroxylation.

In the present paper the crystal structure of the terminal hydroxylase CYP153A*M.aq* has been solved with and without the bound ligand 12‑hydroxy dodecanoic acid (ω‑C12OH). Mutational studies based on this structure have been used to study the determinants of substrate recognition, anchoring and also to alter the enzyme’s selectivity.

Results and Discussion

Crystal structure and identification of structural elements

The structure of CYP153A*M.aq* was determined by molecular replacement using the structure of P450pyr (3RWL, 54% sequence identity)[25] as a model. The structure was determined in two forms: the first with heme in the active site, and the second containing both heme and the ligand 12‑hydroxy dodecanoic acid (ω‑C12OH), representing the first structure of a terminal hydroxylase in the presence of its fatty acid product. The program DALI[26] identified 3RWL as the closest structural homolog, with a Z-score of 53.5 and an rmsd of 2 Å over 404 C‑alpha atoms. Although the full length CYP153A*M.aq* was subjected to crystallisation, residues 1-53 were not visible in the electron density and could not be modelled. A tunnel from the surface of the enzyme to the heme could be identified by the presence of electron density in the omit map, corresponding to ω‑C12OH, in a structure resulting from crystals that had been soaked with this hydroxylation product.(Figure 1) There was continuous linear electron density in the omit map stretching from the heme iron to the active site entrance at approximately 30° from the perpendicular. This could be modelled successfully as 12-hydroxydodecanoic acid (ω‑C12OH) in two conformations, with either the hydroxyl (Fe-OH) or the carboxylate (Fe-COO) of the product coordinated to the heme, as the resolution, at 2.2 Ångstrom, did not permit absolute assignment of those functional groups within the density. The ligand was refined in both orientations, using the same omit map at the same stage of refinement.  The average B-factors of all ligand atoms in the Fe-OH (avge B = 51) structure were slightly higher than those for Fe-COO (avge B = 49) but the average B-factors for the terminal carbon (36) and oxygen (34) atoms over two ligands in Fe-OH were lower than those for the terminal carbon (43) and O atoms (41 and 59) in Fe-COO.  One of the carboxylate oxygens was also not well represented in the refined density in the *F*o-*F*c map in the Fe-COO structure. Figure 1 presents the density for the Fe-OH complex therefore, and this was used as the basis for modelling and structure-guided mutation. Density was observed for the eleven atoms of the carbon chain of ω‑C12OH, but only partially for the carboxylate group of the acid. The long alkyl chain of the product was shown to be accommodated within the hydrophobic tunnel formed by the side chains of L303, I145, A229, M357, I131, T302, G307, T311, F455 and M228 (Figure 2A). The hydroxyl on the terminal carbon was 2.2 Å from the heme iron.

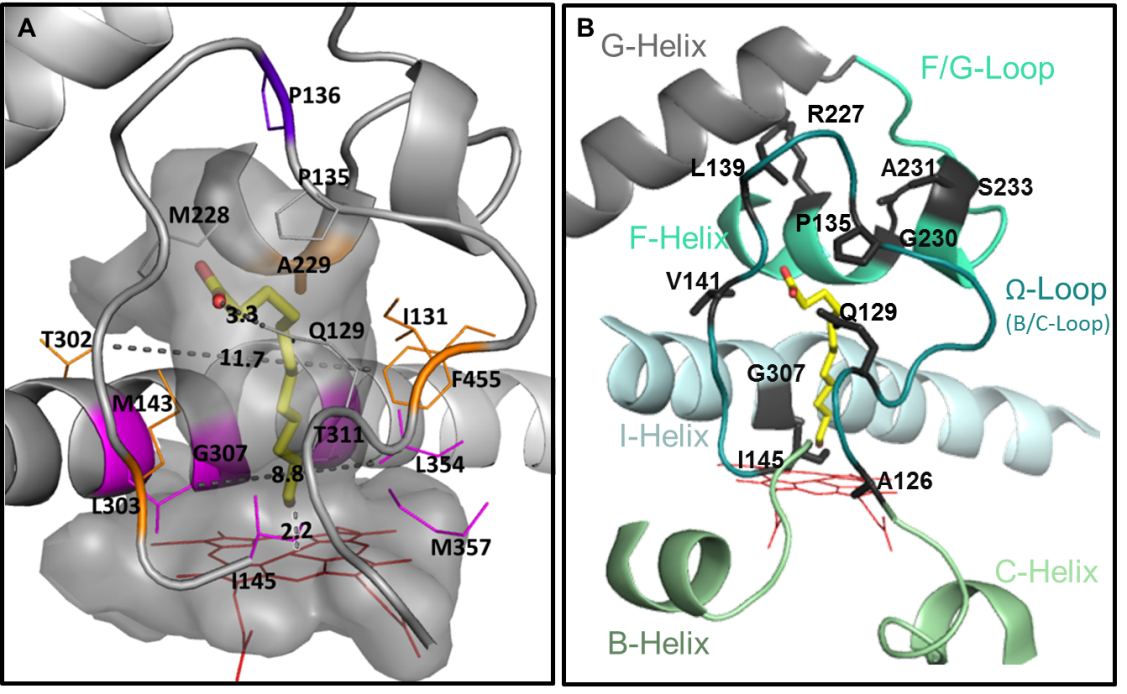


Figure 1. Structure of active site of P450*M.aq,* showing binding of hydroxydodecanoic acid (ω‑C12OH) in the hydrophobic tunnel. Side chains and ω‑C12OH are show in in cylinder format with carbon atoms in green and grey respectively. Heme is shown in ball and stick format with carbon atoms in coral. Electron density corresponds to the 2*F*o-*Fc* (blue)and *F*o-*Fc* omit (green) maps at levels of 1**and 2.5** respectively. The latter is that obtained prior to refinement of ω‑C12OH, the atoms of which have been added from the refined ligand model for clarity. Interaction between the ligand carboxylate and the side chain of Gln129 is indicated by a dashed line with distance is shown in Ångstroms.

The geometry of the binding pocket

The geometry of the binding pocket resembles a truncated cone with a total length of 20 Å from P136 to the heme-Fe. (Figure 2A) The lower tunnel is formed by the amino acid residues of G307, I145, T311, L354 and M357. The lower diameter of the truncated cone is approximately 9 Å and thus a lot narrower than the upper region, which has a diameter of approximately 12 Å. The upper region of the tunnel is formed by T302, F455, A229, M143 and I131. This geometry suggests that the tapering of the tunnel towards the heme is partly responsible for the high selectivity for the terminal carbon.

In previous studies two hotspots, identified from the protein sequence, were the targets of mutagenesis studies.[18] Both are positioned at the bottom of the substrate channel. Mutants at L354 resulted in reduction in ω-selectivity for nonanoic acid, the product distribution of the wt was 97.5 % for ω-OH and 2.5 % for ω-1-OH. When L354 was mutated to isoleucine, 76% of the product mixture was (ω‑1)-OHFA. This again suggested that the tapering structure of the substrate tunnel was necessary to enable terminal hydroxylation. The crystal structure permitted a docking study to support this hypothesis. In the wt, the distance between the terminal C atom of the fatty acid and the heme-Fe is 3.5 Å. In a model of the L354I mutant, the mutation influences the orientation of the substrate in the binding pocket (Figure S1) leading to a twist of the terminal C-atom away from the heme and an increased distance of the terminal C-Atom from the heme-Fe resulting in the terminal hydroxylation being disfavoured.

Similar behaviour was observed in the mutation study of Yang and coworkers.[24] The terminal selective P450pyr was engineered for the subterminal hydroxylation of alkanes. After 6 rounds of directed evolution a mutant was obtained for the hydroxylation of n-octane to give 99 % of (*S*)-2-octanol. The random mutagenesis resulted in an extension of the bottom part of the binding pocket and a horizontal orientation of the substrate.

**Figure 2.** Analysis of the crystal structure. **A** Structure of active site of P450*M.aq*, showing binding of 12-hydroxy dodecanoic acid (ω-C12OH) in the hydrophobic tunnel. Roughly estimated geometry of the binding pocket (gray cavity) resembles a truncated cone which is 20 Å long ((purple) P136 – heme-Fe (red)), to the extended F-helix (A229) the height of the pocket is 13.5 Å. The lower diameter is formed by residues coloured in magenta 9 Å (G307 – M357; I145 – T311; L303 – L354) the upper diameter of the truncated cone is formed by residues coloured in orange 12 Å (T302 – F455; M143 – A229; T302 – I131). The distances in Å are denoted by dashed lines. Interaction between the ligand carboxylate and the side chain of Q129 is indicated by a dashed line with distance is shown in Å.

**B** Active site residues addressed in this study. R227; G230 and A231 at extended F‑Helix coloured in cyan. S233 also located at this structural element was addressed in previous studies. The I‑helix (light blue) is part of the bottom tunnel. The B/C‑loop / Ω**‑**loop is coloured in blue (A126‑I145). The absence of a B’‑helix (as described for CYP102A1) indicates a high flexibility, which is interrupted by P135, further L139 was addressed. Q129 also located at the Ω**‑**loop was predicted for a potential anchor.

From these studies we conclude that for an effective terminal hydroxylation the terminal C-atom has to have a distance around 3 Å to the heme Fe and has to be positioned vertically. The subterminal position has to be fixed and positioned away from the heme by the amino acid residues at the lower region of the tunnel, such as L303 and I145.

Extended F-Helix influences F/G-Loop

The comparison of the crystal structure of CYP153A*M.aq* with known structural elements of P450 enzymes shows some characteristics.[27] The F‑helix of CYP153A*M.aq* (Figure 2B) is extended by a few amino acids which build an additional turn. This extended F‑helix shields the substrate in the active site from the F/G‑loop. This loop is known to interact with the substrate and provides substrate recognition.[28] Furthermore, the additional turn results in a distortion of the alkyl chain of the ligand. Since the natural substrate of CYP153A*M.aq* is not known yet, it is questionable whether this is beneficial for the catalysis of hydroxylation of fatty acids, as the additional turn restricts the length of the substrate tunnel. (Figure 1). The total length of dodecanoic acid in the active site of CYP153A*M.aq* is 13.3 Å. Longer substrates were shown to be poorer substrates for CYP153A*M.aq* .[18] This could be due to the total length of the tunnel. In addition to the correct positioning of the substrate, anchoring in the active site is one of the major steps to allow efficient hydroxylation. According to the crystal structure, there are also no appropriate anchor-sites for the carboxy moieties of longer substrates.

B/C Loop alias Ω Loop

The B/C‑loop (Figure 2B) illustrates the second distinctive feature of the CYP153A*M.aq* structure. The structure of CYP153A*M.aq* in complex with ω‑C12OH suggested that the carboxylate makes contact with the amide side chain of Q129 and the backbone carbonyl group of V141. This leads to the assumption after recognition of the substrate the carboxylate is anchored inside the active site. To study this issue site-directed mutagenesis was performed at this residue 129.

Residue A126 to I145 is part of the B/C‑loop which forms with the tip of the loop (P135 – S140) a part of the active site entrance. The partly absence of E137 and L139 side chains in the electron density indicates flexibility in this part of the loop. Compared to other P450 enzymes as CYP102A1, which is also described as fatty acid hydroxylase, this part of the loop is interrupted by the B’‑Helix.[27] This helix is also lacking in the terminal hydroxylating P450pyr. We suggest that this loop, which we term the Ω‑loop, has to play a crucial role for ω‑hydroxylases.

To study these newly discovered characteristics site‑directed mutagenesis was applied to the above described selected regions.

Structural guided mutagenesis study

Positions at the extended F-Helix (R227, G230, A231), at the Ω‑loop and Q129 – as potential anchor- which is also part of this loop were target of the mutagenesis which are illustrated in figure 2B.

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| **Table 1.** Determination of functional amino acid by rational mutagenesis. | | | |
| Function of mutation site | mutation | Relative conversion of dodecanoic acid[a]  [%] | Relative conversion of hexadecanoic acid[b]  [%] |
| F-Helix | R227A | 107± 5.9 | 108 ± 2.9 |
| G230A | 128 ± 5.6 | 88 ± 2.2 |
| **A231G** | **302 ± 9.8** | 107 ± 3.2 |
| Ω-Loop /  B / C-Loop | L139A | 42 ± 4.2 | 56 ± 3.3 |
| L139Q | 15 ± 4.4 | 48 ± 7.0 |
| L139E | n.c. | 31 ± 8.4 |
| L139R | 19 ± 3.1 | 37 ± 1.2 |
| **P135A** | **142 ± 5.8** | **122 ± 9.9** |
| P135L | 30 ± 0.6 | 79 ± 6.7 |
| P135E | 81 ± 14.8 | 82 ± 3.9 |
| Anchor | Q129A | 83 ± 7.0 | **112 ± 5.9** |
| Q129L | 93 ± 1.5 | n.c. |
| Q129E | *26 ± 1.8* | **114 ± 9.3** |
| **Q129R** | **216 ± 8.2** | 104 ± 5.4 |
| I-Helix | G307A | 105 ± 1.6 | **128 ± 6.7** |
| [a] 1 h reaction time; [b] 2 h reaction; n.c.: no conversion  Conversion of WT was set as 100 %. Relative conversion calculated based on WT conversion of fatty acids detected as hydroxylated product formed per 1.5 µmolP450, 7.5 µM CamA, 15 µM CamB, using a substrate concentration of 200 μM, 2 % DMSO, 30 °C, 750 rpm. | | | |

Mutations within the extended F‑Helix were addressed previously.[22] The exchange of S233 for a glycine led to increased enzyme activity, presumably due to increased space and flexibility. This observation was further studied in this study by generating mutants at A231, G230 and R227. The greatest increase in ω‑hydroxylation was achieved by mutating A231 to glycine, resulting in a 3‑fold improved conversion of dodecanoic acid.(Table 1) The mutation G230A led to reduced activity towards hexadecanoic acid and a slightly improved activity towards dodecanoic acid. The alteration in the largest mutated residue – R227A – showed no influence. However, these observations indicate that this region has a large impact on the effective catalysis and shows different effects on substrate length.

The Ω‑loop was identified as a main characteristic of CYP153. The most flexible part in the Ω‑loop is G138. The density for the neighbouring leucine (L139) is missing for the substrate free structure which indicates high flexibility.

The change of L139 to arginine reduced the activity towards dodecanoic acid to 20 %. No activity was detected for the change to glutamic acid. The introduction of an additional amide side chain through mutation to glutamine at this position did not result in an enhancement of the activity. The opening of the substrate access channel through exchange to an alanine also resulted in a 2-fold reduction in activity relative to the wild type. Leucine at this position seems to have a crucial role. The same tendencies for mutants at position L139 were found for hexadecanoic acid. Proline at position 135 hampers this flexibility and was therefore targeted for the mutation study. To enhance flexibility in the Ω‑loop proline at position 135 was mutated to alanine. This led to a 1.5‑fold enhanced activity towards dodecanoic acid. The insertion of the hydrophobic amino acid leucine resulted in a decreased activity. Flexibility is probably enhanced but the long side chain extends into the narrow pocket and leads to steric hindrance.

Mutants P135L and P135E also reduced the activity toward the longer substrate C16. The enhanced flexibility with mutant P135A resulted also in an enhanced activity toward hexadecanoic acid. 20 % more conversion than the wt were detected.

Q129 is also part of the Ω‑loop. The amide side chain of Q129 was observed to make contact with the carboxylate of the fatty acid. Mutations at this position were made to identify it as potential anchor. Further studies were performed in order to introduce an arginine for better anchoring of fatty acids which has been described for CYP102A1[15] and also to study the anchoring capacity for longer fatty acid substrates.

The substitution of the potential anchoring position Q129 with a smaller (alanine) and a larger (leucine) hydrophobic amino acid did not influence the activity significantly. This suggested that a hydrophobic active site is necessary for effective hydroxylation. Mutation to glutamic acid led to a 5‑fold reduction in activity, which may be explained by repulsion of the negatively charged side chain and the carboxylate moiety of the fatty acid. The opposite was observed for mutant Q129R. The positively charged side chain of arginine may interact with the substrate as described for R47 in CYP102A1[15] and led to a 2‑fold increased conversion. Mutations at position Q129 did not influence the activity towards hexadecanoic acid significantly. However docking studies suggested a close proximity.(Figure S3) Besides anchoring of the substrate in the active site right positioning is the main obstacle of efficient hydroxylation of long chain fatty acids. This may be caused by the extended F/G‑loop, which forces the substrate to bend in the active site. Furthermore, the large hexadecanoic acid does hinder the Ω‑loop flexibility. The introduction of alanine at position 129 and 135 may result in more space and hence increased flexibility. This is reflected by the 12 and 22 %, respectively, increased conversions for these mutants towards hexadecanoic acid.(Table 1)

The 3‑dimensional structure of the active site and substrate orientation helped introducing an anchor Q129R for dodecanoic acid. To further characterise mutant Q129R, kinetic parameters were determined. (Table 2) The *k*cat was not significantly influenced by introducing this mutation, with similar values (6.38 min-1 for Q129R and 6.32 min-1 for wt) measured for each. However, the *K*M value showed a 1.2‑fold reduction, suggesting that the introduction of an arginine side chain had indeed improved the binding of the substrate. This underlines the anchor function.

The overall catalytic efficiency (*k*cat/*K*M) was 1.2-fold increase compared to the wt.

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| **Table 2.** Kinetic parameters of dodecanoic acid for CYP153A*M.aq*variant Q129R and wt with a concentration ratio of 1:5:10 CYP153A*M.aq*:CamA:CamB | | | |
| Mutation | KM  [µM] | kcat  [min-1] | Kcat/KM  [min-1mM-1] |
| Q129R | 67.6 | 6.38 | 94.38 |
| wt | 81.5 | 6.32 | 77.55 |

In order to evaluate any cooperative effect of the mutations, double mutants of G307A, Q129R, P135A and A231G were generated. However, the expression of Q129R/G307A; A231G/G307A and Q129R/A231G resulted in no active P450 enzyme. The double mutant P135A/G307A displayed the same activity towards dodecanoic and hexadecanoic acid as the single mutant. No further improvement in activity could be detected for P135A/A231G.

Conclusions

The first ligand bound structure of an ω‑hydroxylase suggests that a narrow substrate binding pocket is necessary for selective terminal hydroxylation of fatty acids.

An anchor region – where the carboxylate is fixed in the active site – was identified to be residue Q129 by mutational studies and further elucidated by kinetic measurements. The introduced anchor led to a 2‑fold increased product formation and a 1.2‑fold reduced *K*M value.

The major positive effect on catalysis was achieved by extending flexibility in the F‑helix. This helix could be identified to be extended and shields the F/G‑loop from the active site. Loosening this rigid structure by A231G led to a 3‑fold increased activity towards dodecanoic acid.

Distinctive characteristics of CYP153A*M.aq* were identified in the B/C‑loop – herein termed Ω‑loop –. The upper region forms the substrate entrance and is highly flexible. The flexibility of this loop is interrupted in the wildtype by two adjacent prolines. Te flexibility was expanded through the mutation of P135 to alanine. For this mutant, 1.5‑fold increased activity towards dodecanoic acid, and 20 % higher product formation rates with hexadecanoic acid were observed.

Experimental Section

**Site-directed mutagenesis**. Plasmid pET28a(+) harbouring CYP153A*M.aq.*was mutated using the QuikChange© standard protocol to obtain single mutants. Double and triple mutants were obtained by using the single mutant as template. Primers used are listed in the supporting information (Table S2).

Competent *E. coli* DH5α cells were transformed with the *Dpn*I-treated PCR mixtures. Isolated plasmids with the desired mutations (sequencing by GATC-Biotech, Konstanz, Germany) were used to clone the gene into pET28a(+).

The right variants were used to transform competent *E. coli* BL21(*DE3*)

**Expression and quantification.** Freshly plated transformants of *E. coli* BL21 (*DE3*) were grown overnight in LB Medium containing 30 μg mL-1 kanamycin. 1 mL of the pre-cultures was used to inoculate 200 mL TBKan. Cells were incubated at 37 °C on shakers at 180 rpm. At an OD600 of 0.6 – 0.8, cells were induced for recombinant protein expression with 0.1 mM IPTG, 0.1 mM FeCl3 and 0.5 mM δ-aminolevulinic acid. After 16 – 18 h (OD600 = 9-10) incubation at 25 °C cells were harvested by centrifugation (10 000 g, 30 min, 4 °C). Determination of the P450 concentration was carried out by the carbon monoxide (CO) differential spectral assay described by Omura and Sato.[29, 30]

The activity of the variants was assayed *in vitro* by using Putidaredoxin reductase (CamA) and putidaredoxin (CamB) from Pseudomonas putida ATCC17453. Latter support the activity as shown previously.[31, 32] CamA and CamB were expressed and purified as described previously.[33] Protein concentrations were determined spectrophotometrically as described elsewhere.[34]

**Purification.** *E. coli* cells expressing P450*Maq* were disrupted using a Soniprep150 ultrasonicator (MSE, London, UK), employing 3 x 30 s bursts with 30 s intervals at 4°C. The cell debris was then removed by centrifugation, after which the cell-free supernatant was loaded onto a 5 mL His-Trap HP column (GE healthcare). P450*Maq* was purified using a gradient of 0-300 mM imidazole in sodium phosphate buffer (100 mM, pH 7.5) containing 300 mM NaCl. Fractions containing P450*Maq*were pooled, concentrated and subjected to further purification by size exclusion chromatography (SEC) using a HiLoad 16/60 Superdex 75 PrepGrade column (GE Healthcare). Protein was concentrated to 30 mg mL-1 for crystallisation.

**Crystallisation.** Pure P450*Maq* was subjected to commercially-available crystallization screens in 96-well sitting-drop format, in which each drop consisted of 150 nL protein and 150 nL of precipitant solution. The best hits were obtained in 0.1 M bis-tris propane buffer pH 5.5, with 25% (w/v) 3350 polyethylene glycol as precipitant. Larger crystals were obtained using equivalent conditions, but using the hanging-drop method in 24-well plate Linbro dishes, with 2 L drops consisting of 1:1 ratio of precipitant solution to protein. Crystals of the native enzyme were flash-cooled in liquid nitrogen using 15% ethylene glycol as a cryprotectant. For the ω-C12OH complex, crystals were soaked in the mother liquor containing 10 mM ω-C12OH for 1h prior to flash-cooling and testing. Crystals were tested for diffraction in-house using a Rigaku Micromax-007HF fitted with Osmic multilayer optics and a Marresearch MAR345 imaging plate detector. Crystals diffracting to a resolution of equal to, or better than, 3 Å resolution were retained for analysis at the Diamond Light Source synchrotron.

**Data Collection.** Complete datasets described in this report were collected at Diamond Light Source, Didcot, Oxfordshire, U.K. The native P450*Maq* and hydroxydodecanoic acid (ω-C12OH) complexes were collected on beamlines I03 and I04 respectively. Data were processed and integrated using XDS [35] and scaled using SCALA [36] included in the Xia2 processing system [37]. Data collection statistics are given in Table S1**.** All crystals were in spacegroup *P*212121. The structure of P450*Maq* was solved using MOLREP [38],using a monomer model of the P450pyr (PDB code 3RWL) as a model. The solution(s) each contained two molecules in the asymmetric unit, representing one dimer. The structures were built and refined using iterative cycles using Coot [39] and REFMAC [40], the latter employing local NCS restraints. For the ω-C12OH complex of P450*Maq*, following building and refinement of the protein and water molecules, clear residual density was observed in the omit maps in both active sites. This was modelled and refined as ω-C12OH. The final structures exhibited *R*cryst and *R*free values of 19.9 and 23.7 (native); 18.7 and 23.9% (ω-C12OH -complex) respectively. All structures were finally validated using PROCHECK [41]. The coordinates and structure factors for the native and ω-C12OH complexes have been deposited in the Protein Data Bank with the accession codes 5fyf and 5fyg respectively.

**Reaction setup *in vitro*.** *E. coli* BL21(*DE3*) cells were harvested after expression and resuspended in 50 mM potassium phosphate buffer pH 7.5. Cells were disrupted by sonication on ice (4 x 2 min, 2 min intervals). The cell debris was removed by centrifugation (37 000 *g*, 45 min, 4 ***°***C) and the supernatants were recovered.

The activity of each variant was reconstituted with CamA and CamB*in vitro* using C12 saturated fatty acids. Biotransformations were performed using a final volume of 0.15 mL in 50 mM potassium phosphate buffer pH 7.5 containing 1 mM NADPH and the glucose-6-phosphate/glucose-6-phosphate dehydrogenase (G6P/G6PDH) system for cofactor regeneration. For studying the activity and selectivity the different variants were used in a concentration of 3 µM, 7.5 µM CamA and 15 µM CamB. The reaction was started by adding fatty acids in a final concentration of 0.2 mM (from a 10 mM stock solution in DMSO). Samples were incubated at 30 °C and 750 rpm for 1 h**.**

**Analysis of substrates and formed products.** Conversion was stopped with 30 μL 37 % HCl, followed by the addition of internal standard in a final concentration of 0.1 mM (decanoic acid for C12 substrates). The reaction mixtures were extracted twice with 0.4 mL methyl tert-butyl ether. The organic phases were collected and evaporated. Samples were resuspended in 45 μL of 1 % trimethylchlorosilane in *N*,*O*-bis(trimethylsilyl) trifluoroacetamide and incubated at 70 °C for 30 min for derivatisation. Samples were analysed on a GC/MS/FID.

**Determination kinetic parameters.** For the determination of the apparent kinetic constants, 1.5 µM CYP153A*M. aq* plus CamA and CamB (CYP-CamA-CamB in a 1:5:10 ratio), 2% DMSO, 1 mM NADH and cofactor regeneration were used. C12 fatty acids were added in concentrations not exceeding their water solubility limit. Samples were preincubated at 30°C for 2 minutes prior to addition of NADH. Reactions were run at 30°C and 750 rpm and stopped by the addition of HCl after reaction times within the constant rate range (steady-state).

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**Keywords:** terminal hydroxylase • anchor region • P450 • crystal structure • fatty acid

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