Accessing Chemo- and Regioselective Benzylic and Aromatic Oxidations by Protein Engineering of an Unspecific Peroxygenase

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Abstract: Unspecific peroxygenases (UPOs) enable oxyfunctionalisations of a broad substrate range with unparalleled activities. Tailoring these enzymes for chemo- and regioselective transformations represents a grand challenge due to the difficulties in their heterologous productions. Herein, we performed a protein engineering in S. cerevisiae with the novel MthUPO. Experimental approaches were combined with computational modelling resulting in the screening of more than 5,300 transformants. This protein engineering led to a significant reshaping of the active site by elucidated molecular dynamics. The $K_{cat}/K_m$ was improved by 16.5-fold. Variants were identified with high chemo- and regioselectivities in the oxyfunctionalisation of aromatic and benzylic carbons, respectively. The benzylic hydroxylation was demonstrated to perform with excellent enantioselectivities of 95 % ee. Additionally, the first reported effective exchange of the conserved catalytic Glu residue was observed.

Unspecific peroxygenases (UPOs) recently arose as dream biocatalysts for oxyfunctionalisation reactions.[1] They enable the direct transformation of oxygen from hydrogen peroxide to a broad substrate scope capable of reaching impressive total turnover numbers (TONs) beyond 200,000.[2] There is a vast diversity of UPO sequences available within the fungal kingdom with more than 4,300 unique annotated sequences.[3] However, the widespread application and subsequent protein engineering of UPOs are hampered by their challenging heterologous production in fast-growing host organisms.[4] To address these challenges, Alcalde and co-workers[5] as well as our group,[6] developed yeast-based heterologous production setups. These systems gave access to novel UPOs as well as the opportunity for protein engineering. Amongst the recently discovered enzymes, a UPO derived from the thermophilic fungus Myceliophthora thermophila (MthUPO) achieved the thus far highest UPO shake flask production yields. In contrast to the predominantly studied AaeUPO derived from Agrocybe aegerita, the MthUPO revealed the oxidation of naphthalene to 1,4-naphthoquinone as one of two main products.[5-7] These 1,4-naphthoquinone core structures are intensely studied moieties with application in the industry as an intermediate for the anthraquinone synthesis[6] and are widely distributed in the plant and animal kingdom.[9] In nature, naphthoquinones occur in the human body as the blood clotting precursor vitamin K3,[10] in plants as carcinogenesis plumbagin analogues[11] or in insects as chemical defence.[12] We hypothesised that MthUPO’s ability to perform efficient aromatic as well as benzylic oxyfunctionalisations could provide a starting point to engineer chemoselective UPO variants for either specificity. Using the colourimetric assay 5-nitro-1,3-benzodioxole (NBD) [5] around 5,300 transformants were screened to obtain improved variants exhibiting complementary regio- and chemoselectivities as well as improved enantioselectivities. Computational modelling described the molecular basis behind the observed catalytic and selectivity improvements. We commenced our studies by generating a homology model for the MthUPO structure based on the solved crystal structure of the UPO from Marasmius rotula (MroUPO, PDB: 5FUJ, 29% identity and 41 % similarity, see SI for details). The NBD substrate was docked into a representative structure of the most populated cluster, which aided in identifying substrate-binding residues (Fig. 1a). Nine positions (L56, F59, L60, L86, F154, T155, S159, A161, L206, see Fig. S9) were randomised by the Golden Mutagenesis technique.[13] The mutant library was transformed in Saccharomyces cerevisiae producing the corresponding variants. We screened the library using the colourimetric NBD assay[14] in combination with the recently established split-GFP analysis in yeast.[15] The combination of these two assays enabled the distinction between substrate conversion and secretion of the respective enzyme. Variations at position L56 substantially influenced the production of the
enzyme. Only 52% of the transformants displayed a split-GFP signal (Tab. S3). Site saturation at position L60 yielded improved variants with superior TONs for NBD conversion. The variants L60M (1.2-fold relative to the wildtype), L60Q (1.3-fold) and L60F (2.7-fold) showed the most noticeable improvements (Tab. 1 and S4). Variant L60F also exhibited a 1.4-fold improved catalytic efficiency ($k_{\text{cat}}/K_m$, Tab. 2). The variant library of position F154, which is located at the entrance channel, turned out to be a pivotal position for the NBD conversion. Even though 81% of the variants were secreted according to the split-GFP signal, only the rediscovered wildtype enzymes displayed activity (Fig. S1). To gain insights into the highly conserved catalytic residue E158, we tested the variants E158A and E158D. E158A led to the expected loss of NBD activity. To retain the deprotonation of the peroxo-iron complex, we introduced an aspartate as a shortened amino acid.

Table 1. The catalytic activity of MthUPO variants for the hydroxylation of NBD.[4]

| Catalyst | Conversion [%] | TOF [min⁻¹] | TON |
|----------|----------------|--------------|-----|
| MthUPO WT | 29 | 72 | 4340 |
| MthUPO L60F | 77 | 194 | 11610 |
| MthUPO E158D[6] | 44 | 6 | 6560 |
| MthUPO H88A/E158D[6] | 32 | 16 | 970 |
| MthUPO L60F/S159G/A161F | 84 | 210 | 12590 |
| MthUPO F59G/L60M/S159G/F154A | 77 | 192 | 11540 |
| MthUPO F59G/L60F/S159G[4] | 76 | 379 | 22760 |

TOF = turnover frequency, TON = turnover number, standard deviation < 3.2%.

Astonishingly, variant E158D proved to be active with a decelerated but prolonged product formation. Whereas the MthUPO wildtype reached the maximum product formation after a few minutes, the variant E158D converted the substrate overnight yielding 1.5-fold improved TONs relative to the wildtype. This variant represents the first exchange of the catalytic glutamate residue while preserving the UPO activity. Computational analysis based on MD simulations revealed that the E158D mutation induces higher rigidity to the $\alpha$-helix than the wildtype (Figs. S10 and S11), which might be responsible for its prolonged conversion.

The 23.6-fold decreased $k_{\text{cat}}$ value of variant E158D (Tab. 2) indicates that the mutation has a severe impact on the rate-limiting step of the reaction. This impact is most likely related to the $\text{H}_2\text{O}_2$ activation via proton transfer steps that are mediated by this residue.[16] To assess the influence of the peroxide species with these variants, we employed cumene hydroperoxide and tert-butyl hydroperoxide as oxygen source. These experiments revealed diminished activities using the alternative peroxides with the wildtype and E158D and no activity with E158A (Fig. S3).

The catalytic glutamic acid residue in UPOs is well known to coordinate to histidine or arginine stabilising the negative charge formed at the acid-base catalyst.[1,17] MD simulations proved that $\text{H}_8\text{H}_8$ can establish persistent H-bond interactions with both E158 and H88 by generating the variants H88A and H88A/E158D. Variant H88A led to no product formation while variant H88A/E158D was able to convert NBD with a 4.5-fold decreased TON relative to the wildtype. MD simulations indicated that in the absence of H88 (H88A) the $\alpha$-helix becomes even more flexible and disordered, whereby the side chain of position E158 is barely preorganised to activate $\text{H}_2\text{O}_2$. On the other hand, the shorter side chain of E158 in H88A/E158D is still well-positioned to generate compound 1 (Fig. S12).

To continue with the mutagenesis approach, we grouped two amino acid residues and saturated them simultaneously with a reduced codon degeneracy (NDT), thereby obtaining double mutants with improved TONs (Tab. S4). The improved double mutated variants were L60F/F154I and L60F/F154V (1.2-fold improvement compared to the wildtype, Tab. S4).

![Figure 1. Active site arrangement of MthUPO and evolution of NBD’s catalytically relevant binding modes in a) wildtype and the variants b) L60F and c) L60F/S159G/A161F as observed from MD simulations. Mutated positions are highlighted in orange.](image-url)
Whereas the recombination library resulted in the discovery of triple and quadruple mutations with up to 16.5-fold improved catalytic efficiency (kcat/Km, Tab. 2). All of the most active variants harboured amino acid exchanges at the L60 position (L60F/M) and the mutation S159G. The kinetic measurements revealed 8.2-fold (F59Q/L60F/S159G), 10.8-fold (F59Q/L60M/S159G/F154A) and 16.5-fold (L60F/S159G/A161F) increased kcat/Km values relative to the wildtype (Tab. 2). Whereas the Km value of NBD was decreased or similar to the wildtype, the values were significantly decreased for H2O2 (Tab. S5). The kcat for NBD, however, was substantially improved for the identified triple and quadruple mutants with an 18.6-fold increase for the variant L60F/S159G/A161F relative to the wildtype. Docking calculations were used to obtain starting points for MD simulations in order to analyse the binding of NBD in the wildtype and the variants L60F and L60F/S159G/A161F (Figs. 1, S13 and S14). These simulations revealed a switch in the binding mode of NBD when moving from wildtype to L60F and L60F/S159G/A161F (Fig. S13). Due to the inclusion of bulkier residues in the inner active site (first L60F, then A161F), the NBD substrate was reoriented from its original more buried binding pose in the wildtype to partially occupying the entrance channel in L60F/S159G/A161F. This new binding pose allows the NBD to better approach the Fe=O active species in a near attack conformation, thus facilitating the oxidation reaction (Fig. S14).

To determine the influence of the engineered variants on the regio- and chemoselectivity, we investigated the oxyfunctionalisation of naphthalene and its derivatives (Scheme 1). The most active variants were selected, and an initial screening was performed by GC-MS, resulting in significant changes in the selectivity (Fig. S6-S8). Utilising a syringe pump setup achieved more than 10,000 TONs with variant F59Q/L60F/S159G for the conversion of naphthalene to 1,4-naphthoquinone 1a (Scheme 2a). These TONs represent a 10-fold increase to the previously published MthUPO wildtype activity.16 By increasing H2O2 equivalents, 2-hydroxy-1,4-naphthoquinone 1b was formed as a by-product, which is a natural dye known as Lawsone.19

The biotransformation of 2-methylnaphthalene with the wildtype led predominantly to 6-methyl-1,4-naphthoquinone (Fig. S7). Variant L60F/S159G/A161F was able to shift the major product formation to 2-methyl-1,4-naphthoquinone

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**Table 2.** Biochemical characterisation of the MthUPO wildtype and the evolved variants towards the substrate NBD.11

| Catalyst          | Tm [°C] | Km [µM] | kcat [s⁻¹] | kcat/Km [M⁻¹s⁻¹] |
|-------------------|---------|---------|-------------|-------------------|
| MthUPO WT         | 63.5    | 386     | 7.1         | 1.9               |
| MthUPO L60F       | 55.7    | 110     | 2.9         | 2.7               |
| MthUPO E158D      | 60.8    | 186     | 0.3         | 0.1               |
| MthUPO            | 56.9    | 422     | 132.2       | 31.3              |
| L60F/S159G/A161F  | 54.7    | 290     | 59.4        | 20.5              |
| MthUPO            | 55.8    | 303     | 47.0        | 15.5              |
| F59Q/L60M/S159G/F154A | 55.8 | 303 | 47.0 | 15.5 |
| MthUPO            | 55.8    | 303     | 47.0        | 15.5              |
| F59Q/L60F/S159G   |         |         |             |                   |

Standard deviation <18 %, [a] Values were calculated with the corrected extinction coefficient of 10870 M⁻¹cm⁻¹, reaction conditions and Michaelis-Menten plots: see Supporting Information.

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**Scheme 1:** Catalytic activity of MthUPO variants for the hydroxylation of naphthalene and its derivatives (reaction conditions are shown in Tab. S6, standard deviation <5.2 %)
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(2a), also known as vitamin K3, demonstrating the regioselectivity of this variant (Scheme 2b). We were pleased to note that we could also identify a variant with the preference for the methyl hydroxylation of 2-methylnaphthalene. Variant L60F showed an altered chemoselectivity dramatically suppressing the aromatic hydroxylation and accessing the hydroxylation of the methyl group (2b) as the major product. Also the overoxidation to the aldehyde was observed (2c, Scheme 2b). MD simulations with the variant L60F and 2-methylnaphthalene demonstrated that only the methyl group was able to approach the active Fe=O species in a catalytically competent pose due to the presence of the bulky phenylalanine residue L60F (Fig. S15). When 2-methylnaphthalene was bound into the variant L60F/S159G/A161F (Fig. S16), however, a binding pose similar to the previously observed for NBD in this variant was observed (Figs. 1 and S13). This binding mode is promoted by hydrophobic interactions occurring in the newly engineered active site, which is dominated by the presence of several aromatic residues (F59, F60, F63, F154, and F161). Within this new binding pose, the substituted aromatic ring 1 of 2-methylnaphthalene is placed close enough to the Fe=O catalytic species to react while keeping the 2-methyl group away from it. This different binding mode of the L60F variant is responsible for the observed switch in chemoselectivity (Figs. S16 and S17).

When the methyl substitution was changed from 2-methyl to 1-methylnaphthalene a decrease in the activity of 50 % was observed yielding to the opposite unsubstituted ring 2 oxidation (Scheme 2c). More surprisingly, also substitutions in position 2 at the naphthalene led to the oxidation of the unsubstituted ring generating the products 6-methoxy- (4a, Scheme 2d) and 6-bromo-1,4-naphthoquinone (5a, Scheme 2e) with diminished TONs relative to 2-methylnaphthalene. For 2-bromo-naphthalene also 2-bromo-1,4-naphthoquinone (5b) was detected as a by-product. MD simulations with variant L60F/S159G/A161F and 2-methoxy-naphthalene (Fig. S18) revealed a preferential binding in a catalytically competent pose equivalent to the binding mode observed for 2-methyl naphthalene (Fig. S17). However, the bulky methoxy group at the 2-position sterically clashes with the L60F residue, and the naphthalene core needs to rotate slightly when approaching the Fe=O active species to place the 2-methoxy group away from the L60F and the heme cofactor. This rotation brings the unsubstituted aromatic ring 2 closer to the Fe=O active species, as opposite to 2-methylnaphthalene (Fig. S17), inducing a shift in the regioselectivity (Fig. S18). A similar behaviour was also observed for 1-methylnaphthalene when bound in variant L60F/S159G/A161F in a catalytically competent pose (Fig. S19).

Intrigued by the chemoselective benzylic hydroxylation of L60F, we tested this variant with indane and tetralin (1,2,3,4-tetrahydronaphthalene). L60F was able to convert indane with more than 8,000 TONs and an improved enantioselectivity for the (R)-1-indanol (5a) from 85 % ee (wildtype) to 95 % ee (Tab. 3 and S7). Interestingly, further examination of the most active variants L60F/S159G/A161F and F59Q/L60M/S159G/F154A revealed the excess in formation of the (S)-enantiomer (Tab. S7). For the bioconversion of tetralin to the alcohol 6a, an improved enantioselectivity for the (R)-enantiomer was detected for L60F (74 % ee) relative to the wildtype (45 % ee). Similar to indane, a variant could be identified, which forms predominantly the (S)-enantiomer (F59Q/L60M/S159G/F154A). The overoxidation to the ketone as a major product was achieved by the same variant with TONs of 440.

MD simulations with indane bound within the L60F variant (Fig. S20) characterised a preferential binding pose of the substrate that resembles the previously observed pose for NDB and 2-methylnaphthalene in L60F/S159G/A161F (Figs. S13 and S16). In this case, indane mainly interacts with the aromatic rings of F63 and L60F, establishing C-H...π interactions. These hydrophobic interactions keep the substrate in a preferred binding pose where only the pro-R 1-H-indane is close enough and well-aligned to the Fe=O active species to be efficiently hydroxylated (Fig. S20).

Table 3. The catalytic activity of MthUPO variants towards benzylic hydroxylation yielding chiral products.\(^d\)

| Table 3. The catalytic activity of MthUPO variants towards benzylic hydroxylation yielding chiral products.\(^d\) |
|---|---|---|---|---|
| R | Catalyst | Product | TON | % ee\(^e\) |
| CH\(_2\) | MthUPO L60F\(^d\) | \(\text{H}_2\text{O}_2\) | KPi pH 7 | 8160 | 95 (R) |
| CH\(_2\) | MthUPO L60F\(^d\) | \(\text{H}_2\text{O}_2\) | KPi pH 7 | 450 | - |
| (CH\(_2\)\(_2\)) | MthUPO L60F\(^d\) | \(\text{H}_2\text{O}_2\) | KPi pH 7 | 860 | 74 (R) |
| (CH\(_2\)\(_2\)) | MthUPO F59Q/L60M/S159G/F154A\(^d\) | \(\text{H}_2\text{O}_2\) | KPi pH 7 | 440 | - |

TON = turnover number, standard deviation < 6.5 %, reaction conditions: \(a\) 100 nM MthUPO variant, 1 mM indane, 1 mM\(\text{H}_2\text{O}_2\), 100 mM KPi buffer (pH 7), 5 % acetone \((\text{v/v})\), 1 h at 25 °C in triplicates, \(b\) 100 nM MthUPO variant, 1 mM 1,2,3,4-tetrahydronaphthalene, 1 mM\(\text{H}_2\text{O}_2\), 100 mM KPi buffer (pH 7), 5 % acetone \((\text{v/v})\), 1 h at 25 °C in triplicates, \(c\) 100 nM MthUPO variant, 1 mM 1,2,3,4-tetrahydronaphthalene, 2 mM\(\text{H}_2\text{O}_2\), 100 mM KPi buffer (pH 7), 5 % acetone \((\text{v/v})\), 2 h at 25 °C in triplicates, \(d\) determined by chiral GC.
In the present work, the novel MthUPO was engineered using S. cerevisiae as expression host and NBD as an assay system. By initial saturation of relevant amino acid residues in single and double saturation mutageneses, key residues were identified. This mutagenesis also led to an increased understanding of the highly conserved catalytic glutamate. Its stabilisation by histidine, and—most surprisingly—the generation of the active variant E158D. A recombination library of the best performing variants revealed substantially improved variants with triple and quadruple mutations. Variant L60F demonstrated a high chemoselectivity for the oxidation at the benzylic position, whereas L60F/S159G/A161F selectively formed aromatic ring oxidations with 2-methylnapthalene. L60F also revealed an improved enantioselectivity up to 95% ee for the conversion of indane to its benzylic hydroxylated derivative. MD simulations described a reshaping of MthUPO’s active site during evolution due to the inclusion of bulkier residues, which induced novel binding modes for the studied substrates, and rationalised enzyme-substrate specific interactions providing the molecular basis for the experimentally observed oxidation patterns. These results demonstrate the high versatility and evolvability of MthUPO to achieve regio-, chemo- and enantioselective biocatalytic oxycarbonylation.

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