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Switching the Regioselectivity of a Cyclohexanone Monooxygenase toward (+)-*trans*-Dihydrocarvone by Rational Protein Design

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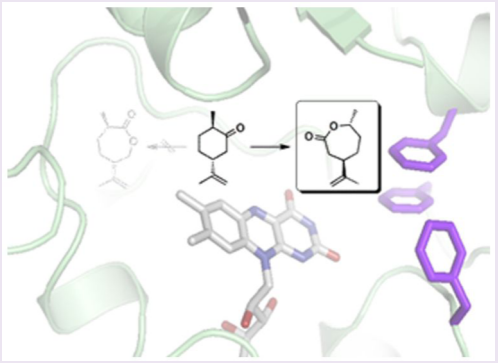
Supporting Information

ABSTRACT: The regioselectivity of the Baeyer–Villiger monooxygenase-catalyzed oxidation is governed mostly by electronic effects leading to the migration of the higher substituted residue. However, in some cases, substrate binding occurs in a way that the less substituted residue lies in an antiperiplanar orientation to the peroxy bond in the Criegee intermediate yielding in the formation of the "abnormal" lactone product. We are the first to demonstrate a complete switch in the regioselectivity of the BVMO from *Arthrobacter* sp. ($CHMO_{Arthro}$) as exemplified for (+)-*trans*-dihydrocarvone by redesigning the active site of the enzyme. In the designed triple mutant, the substrate binds in an inverted orientation leading to a ratio of 99:1 in favor of the normal lactone instead of exclusive formation of the abnormal lactone in case of the wild type enzyme. In order to validate our computational study, the beneficial mutations were successfully transferred to the CHMO from *Acinetobacter* sp. ($CHMO_{Acineto}$), again yielding in a complete switch of regioselectivity.



Baeyer–Villiger monooxygenases (BVMOs) belong to the FAD-dependent oxidoreductases and catalyze the enzymatic pendant of the chemical Baeyer–Villiger oxidation. An oxygen atom is inserted next to a keto function yielding in the formation of esters or lactones. BVMOs have been studied since the 1950s[1] and have often shown excellent stereo- and chemoselectivity while offering a wide substrate spectrum.[2,3] This broad applicability and the fact that enzymatic Baeyer–Villiger oxidations are much more environmentally and economically friendly due to the use of molecular oxygen instead of peracids or transition metal catalysts make BVMOs valuable catalysts. The conversion of asymmetric ketones with BVMOs can lead to the formation of two regio-isomeric products. When the oxygen atom is inserted next to the more nucleophilic and higher substituted residue, the product is called the "normal" lactone/ester. When the oxygen atom is inserted next to the smaller residue, the "abnormal" product is formed. These regiodivergent conversions using different BVMOs have been mostly reported for fused bicyclic ketones.[4−7] Additionally, it was shown that some aliphatic substrates like certain β-hydroxyketones and β-aminoketones can be converted to abnormal esters.[8−11] Recently, the formation of abnormal product was reported for the conversion of methyl ethyl ketone by different BVMOs. It was found that an excess of the substrate led to a better ratio of abnormal over normal product.[12] However, in many cases, the enzymes did not exhibit a perfect regioselectivity, and both possible regio-isomers were formed, yielding a mixture of products. In those cases, it is mandatory to understand how regioselectivity is controlled by the enzyme so that only the desired product can be obtained. The most widely accepted assumption on how regioselectivity is controlled by the enzyme is based on the structure and the reordering of the Criegee intermediate, which is formed in the course of the reaction and leads to the release of the product. The migrating bond in the Criegee intermediate is supposed to be antiperiplanar to the oxygen–oxygen bond of the peroxide. This assumption was investigated and confirmed for the conversion of bicyclic substrates by the cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp.[4] Additionally, the preferred conformation of cyclohexanone and 4-methylcyclohexanone in the active site of the CHMO from *Rhodococcus* has been rationalized in a QM/MM study showing that only one of the two possible σ bonds adjacent to the keto function of the substrate is in a conformation, which ensures maximum σ orbital overlap. This finding made it possible to rationalize the experimentally observed enantioselectivity of the enzyme toward 4-methylcyclohexanone and might also facilitate the prediction of regioselectivity.[13] Apart from the prediction of regioselectivity, in practical experiments it has been observed that the amount of abnormal product can also be slightly influenced by the oxygen supply.[9,10]

The aim of this study was to understand how regioselectivity can be controlled and to ascertain whether it is possible to switch the regioselectivity. We used the conversion of (+)-*trans*-dihydrocarvone by the CHMO from *Arthrobacter* sp. BP2 ($CHMO_{Arthro}$)[14] as a model reaction. This reaction was already investigated in more detail,[15] and it was reported that $CHMO_{Arthro}$ converts (+)-*trans*-dihydrocarvone exclusively to

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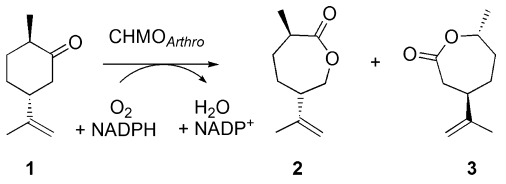
the abnormal lactone (Scheme 1). Changing the regioselectivity of the enzyme toward this substrate so that only the normal

Scheme 1. Conversion of (+)-*trans*-Dihydrocarvone (1) by $CHMO_{Arthro}$ to the Abnormal (2) and Normal (3) Lactone[a]



[a]Note: The wild type enzyme exclusively yields product **2**.

lactone is produced would not only give valuable insights into the mechanism of this reaction but would also allow access to the normal (4*R*,7*R*)-7-methyl-4-isopropenyl-2-oxo-oxepanone enantiomer, which cannot be selectively produced by any of the BVMOs investigated by Cernuchova and Mihovilovic.[15]

In order to change the regioselectivity of the enzyme toward the *trans*-dihydrocarvone isomer by rational protein design, it was mandatory to understand the mechanism of this reaction and to have a reliable structural model of this enzyme. Since there is no crystal structure of this BVMO available, it was essential to find a homologous enzyme, which has been characterized in more detail. The most extensively studied BVMOs are the CHMO from *Acinetobacter* sp. ($CHMO_{Acineto}$) and the phenylacetone monooxygenase from *Thermobifida fisca* (PAMO). In 2004, the crystal structure of PAMO was published as the first available BVMO structure.[16] Even though the $CHMO_{Acineto}$ has been investigated in many studies and offers a wide substrate spectrum,[17−20] the crystal structure of this enzyme has not been solved until now. This is probably mainly due to the poor stability of this enzyme. However, a close homologue of this enzyme, the CHMO from *Rhodococcus* sp. HI-31, was successfully crystallized by Mirza et al.[21] This enzyme shows 84% homology to $CHMO_{Arthro}$. In the study from 2009, two structures with bound FAD and $NADP^+$ were presented ($CHMO_{Open}$ and $CHMO_{Closed}$; PDB codes: 3GWF and 3GWD). Further crystal structures of this enzyme in combination with substrate ($CHMO_{Rotated}$; PDB code: 3UCL)[22] and product ($CHMO_{Tight}$ and $CHMO_{Loose}$; PDB codes: 4RG3 and 4RG4)[23] followed. These structures of the CHMO from *Rhodococcus* allow insights into different steps of the mechanism and facilitate the interpretation of dynamic movements of BVMOs during catalysis. By preparing homology models based on those structures and the identification and mutation of relevant amino acids, we show here that we were able to identify a triple mutant that offers a complete switch of the regioselectivity in the conversion of (+)-*trans*-dihydrocarvone, yielding 99% normal product. As the CHMO from *Acinetobacter* sp. has a similar active site to that of $CHMO_{Rhodo}$ and $CHMO_{Arthro}$, the beneficial mutations were introduced in this enzyme in order to validate the value of our computational design. The $CHMO_{Acineto}$ wild type enzyme shows the same characteristics in the conversion of (+)-*trans*-dihydrocarvone as the $CHMO_{Arthro}$ wild type, and the generated triple mutant indeed also resulted in a complete switch in regioselectivity.

When we started our studies only the $CHMO_{Open}$, $CHMO_{Closed}$, and $CHMO_{Rotated}$ structures as described *vide supra* were available. In the beginning, we built $CHMO_{Arthro}$ homology models based on the *closed* and *rotated* structures as those seemed to be mechanistically relevant. Yachnin et al. assumed that the enzyme is in the *open* conformation in the beginning of the reaction, when molecular oxygen from air reacts with the reduced flavin cofactor to form the C4α-peroxyflavin intermediate.[22] After the substrate has entered the active site, the enzyme will change to the *closed* conformation. The positioning of the substrate binding would then be decided in the *closed* conformation. As the NADPH blocks the interaction of the substrate and FAD/peroxy group in the *closed* conformation, the NADPH moves away from the FAD to yield the *rotated* conformation. In this conformation, the substrate is guided closer to the peroxy group with the help of Arg329 ($CHMO_{Rhodo}$ numbering) so that the Criegee intermediate can be formed. The subsequent rearrangement of the Criegee intermediate is controlled by the directionality of the enzyme. The enzyme probably exhibits a certain directionality when inserting the oxygen into the substrate so that the oxygen will always be entered at the same position.[23] Only the orientation of the substrate will then decide which enantiomer or regio-isomer is formed. Following the rearrangement of the Criegee intermediate, the corresponding lactone product, water, and oxidized FAD are released while the enzyme changes first from the *rotated* to the *closed* and then the *open* conformation. This, by Yachnin et al., first proposed order of the different enzymatic movements as derived from the different crystal structures complemented the mechanism of this reaction, which was first postulated in 1982[24] and gave valuable insights into the structural movements of CHMOs during the mechanism.

When converting asymmetric ketones, the two regio-isomers are formed by different binding modes during the formation of the Criegee intermediate. So, in order to predict and change regiospecificity by rational protein design, the conformation of the substrate in the *closed* structure and the *rotated* structure are of main importance. The binding of the substrate in the *closed* conformation determines the orientation of the substrate. When looking at the substrate position in the *rotated* conformation, the structure of the Criegee intermediate can be predicted, and thus it should be possible to determine which bonds are in an antiperiplanar orientation and thus which regio-isomer will be formed. In our study, we used YASARA[25−28] for computational modeling. Based on the $CHMO_{Arthro}$ *closed* and *rotated* homology models, 16 amino acids presumably necessary for substrate positioning were identified. Thirteen of these relevant amino acids were mutated to alanine in order to evaluate how important they actually are for substrate positioning and catalysis (data for all mutated amino acids can be found in the Supporting Information; Table 1). The active site residues R380, W543, and D110 corresponding to the amino acids R329, W492, and D59 in the $CHMO_{Rhodo}$ structure were not mutated, as those were already shown to be relevant for catalysis,[16,21] and the W492A mutation led to significantly decreased activity in an earlier study.[21] The mutation of R380 ($CHMO_{Arthro}$ numbering) to alanine would not be reasonable as this residue has been shown to be crucial for substrate positioning.[22] Additionally, R329 and D59 seem to be relevant for the positioning of $NADP^+$.[16,21,29] The proposed enzyme variants were successfully expressed in *E. coli* BL21(DE3). Whole cells were used for biocatalysis reactions with (+)-*trans*-dihydrocarvone as a substrate. From this first round of mutagenesis, we identified the $CHMO_{Arthro}$ variant F299A, which gave 59% normal lactone instead of 100% abnormal lactone formation produced by the wild type enzyme. The only other enzyme variant, which showed good activity

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Table 1. Overview of the Generated CHMO$_{Arthro}$ Variants Comparing Substrate Conversion and Ratio of Normal to Abnormal Product

enzyme variant	conversion of **1** [%]	normal/abnormal
wild type	100	0:100
L196F/F299A/F330A	14	96:4
L197F/F299A/F330A	19	92:8
F299A/F330A/F485A	39	99:1
F299V/F330A/F485A	37	99:1
F299A/F330A/F558V	12	95:5
F299V	39	74:26
F330V	70	0:100
F299A/F330V	43	69:31
F299V/F330A	18	91:9
F299V/F330V	29	75:25

and a slight change in regioselectivity was F330A with about 3% normal lactone. The corresponding double mutant was generated, which resulted in the production of 86% normal lactone in whole cell biocatalysis. At this point the CHMO$_{Tight}$ structure was released and promised to be a better scaffold for rational protein design when aiming at changing the enantio- or regioselectivity.[23] Therefore, homology models based on the CHMO$_{Rhodo}$ *tight* conformation were built for CHMO$_{Arthro}$ wild type, F299A, and F299A/F330A. As this structure was published with bound product, docking experiments with the two possible products could now be performed in order to determine the different positioning of those products in the variants. We assumed that the directionality of CHMO$_{Arthro}$ might be the same as for CHMO$_{Rhodo}$ due to the close structural resemblance of the active sites. This way we were able to compare our docking results with the product conformation as shown in the CHMO$_{Tight}$ structure and identified the product orientations of the two possible (+)-*trans*-dihydrocarvone products. In the wild type structure, the abnormal product is tilted in a way that the two product residues are in the same plane as the ring (Figure 1A). Consequently, they are not colliding with any of the amino acid residues in the active site. As expected, we did not get a convincing docking result from the wild type structure with the normal lactone. However, the normal product can be docked into the F299A mutant structure (Figure 1B). In this variant, the lactone is not tilted sideways because the larger residue would then collide with L196. Nevertheless, from the docking result, it was clear why this single mutation would not exclusively lead to normal lactone formation: the larger substrate residue is very close to the residues W543 and F558 so that the binding of the substrate in this orientation would not be favored. Furthermore, it does not become evident why the F299A single mutation makes normal lactone binding possible as the general positioning of the abnormal lactone in the wild type structure and that of the normal lactone in the single mutant are very similar. However, the methyl group of the normal product in the F299A/F330A docking experiment would interfere with the phenylalanine side chains in positions 299 and 330 (Figure 1C). In this structure, the product is tilted again but moved away from the middle of the active site. The larger residue of the product is in the same plane as the ring of the abnormal product in the wild type structure when superposing those structures.

In order to achieve exclusively normal product formation, we assumed that we either have to open up the space above the substrate so that the larger side chain can move up more or have to close up the space where the large residue is positioned in the abnormal product binding. With this strategy in mind, we

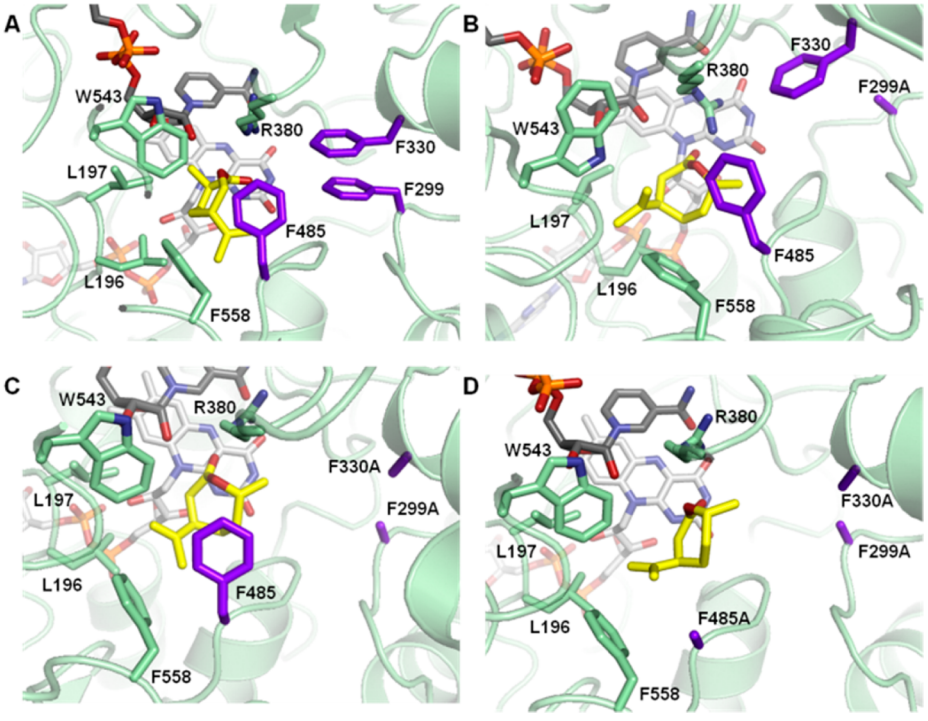

Figure 1. Comparison of the results of the docking experiments with the lactone product for CHMO$_{Arthro}$ *tight* structures. (A) Docking experiment of the abnormal lactone in the CHMO$_{Arthro}$ wild type structure, docking experiments with the normal lactone in (B) the F299A variant, (C) the F299A/F330A variant, and (D) the F299A/F330A/F485A variant. Protein is shown in green, mutated key residues in purple. Cofactors are depicted in gray (FAD in light gray and NADPH in dark gray). The lactone product is shown in yellow.

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prepared four triple mutants in order to switch the regioselectivity completely. Variants with the L196F or L197F mutation were designed in order to impede the usual substrate binding for the formation of the abnormal product. The mutation of positions F485 and F558 to smaller residues facilitates the binding of the substrate in a way that the normal product will be formed as the active site is opened up and room is created for the large substrate residue. All those mutants showed an increase in normal lactone production when used in whole cell biocatalysis reactions compared to the double mutant (Table 1). Variant F299A/F330A/F485A showed the desired complete switch in regioselectivity as 99% normal lactone was formed in small-scale whole cell biocatalysis instead of 100% abnormal lactone in the case of the wild type enzyme.

Additionally, we prepared the variants F299V, F299A/F330V, F299V/F330A, and F299V/F330V in order to determine whether a slightly larger residue at these positions has an influence on the regioselectivity. It was observed that a valine at position F299V increases the amount of normal lactone when compared to the corresponding alanine mutants (Table 1). On the contrary, a valine in position 330 seems to be too large since the F330V single mutant shows the same regioselectivity as the wild type and the F229V/F330V the same as the F299V variant. Thus, the mutation of F330 to alanine is crucial for the change of regioselectivity, but it only has an effect in combination with the mutation of F299 to a smaller residue. The variant F299V/F330A/F485A showed the same characteristics as F299A/F330A/F485A.

In order to compare the substrate binding in the different variants, another docking experiment with F299A/F330A/F485A was performed. The F485A mutation indeed allowed the large product residue to point upward more than in the double mutant (Figure 1D). Once the wild type and F299A/F330A/F485A docking experiments are superposed, it is obvious that the larger residue would collide with F485. Additionally, the methyl group would clash with F330. Mutations of F558 to alanine or valine led to an increase of the amount of normal lactone, but those variants showed an even more significant decrease in activity.

In order to prove the transferability of the computational design, the three beneficial mutations found for $CHMO_{Arthro}$ were introduced into $CHMO_{Acineto.}$ These two enzymes have similar active sites but the overall homology is only 57%. The corresponding amino acids in the $CHMO_{Acineto}$ active site were mutated, yielding the F246V/F277A/F432A $CHMO_{Acineto}$ variant. The regioselectivity of this variant was compared to the $CHMO_{Acineto}$ wild type enzyme for the conversion of (+)-*trans*-dihydrocarvone in a biocatalytic reaction. Both enzymes showed full conversion after 48 h reaction time, but while the wild type enzyme gave 97.6% abnormal lactone, the substrate conversion using the triple variant yielded in 99.6% normal lactone and thus again resulted in a complete switch in regioselectivity.

In order to determine optimal conditions for reactions with the $CHMO_{Arthro}$ variants, the wild type enzyme was characterized in more detail. Kinetic parameters were determined for (+)-*trans*-dihydrocarvone as a substrate (Table S2).

As shown in the Michaelis–Menten kinetic (Supporting Information Figure 1), the enzyme is already inhibited at low substrate concentrations. Significant substrate inhibition can be observed at substrate concentrations above 6 mM. For that reason, we decided to use 6 mM as the highest substrate concentration in biocatalysis reactions. Furthermore, the wild type enzyme was characterized concerning optimal pH and temperature as well as temperature stability. The optimal pH is 7.3 (Supporting Information Figure 2A) and the optimal temperature 43 °C (Supporting Information Figure 2B). Temperature stability was determined by incubating the enzyme at 20, 25, and 30 °C and measuring the activity at certain time points. The enzyme is completely inactivated after about 4 h at 30 °C. At 25 °C, the enzyme still has 50% of its initial activity after 4 h, and at 20 °C, the enzyme still exhibits half of the initial activity after 43 h (Supporting Information Figure 2C). On the basis of these results, we decided to perform all biocatalytic reactions at 20 °C. In order to compensate for the fact that the enzyme exhibits only 55% of its potential activity, the biocatalytic reactions were performed for at least 24–48 h. After checking the regioselectivity of the enzyme variants in triplicate small scale biotransformations, duplicates on a larger scale were performed as well as biocatalysis reactions using purified enzyme. In Figure 2, a comparison of the regioselectivities of the best enzyme variants as observed in small-scale biocatalysis is shown.

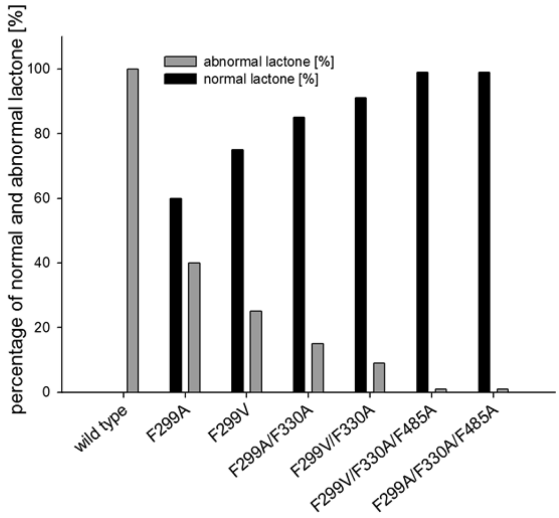

Figure 2. Comparison of the amount of normal and abnormal lactone produced from (+)-*trans*-dihydrocarvone by $CHMO_{Arthro}$ variants in small-scale whole cell biocatalysis reactions.

The triple variant F299A/F330A/F485A was investigated and compared to the wild type enzyme by taking time point measurements of a biocatalysis prepared on a larger scale. Full conversion of 2 mM (+)-*trans*-dihydrocarvone was achieved after 1 to 1.5 h in a whole cell biocatalysis with the wild type and after 4 to 5 h, yielding 98% normal lactone with the triple variant (Supporting Information Figure 3A). Biocatalysis reactions at 6 mM (+)-*trans*-dihydrocarvone gave 97% conversion after extended reaction times, yielding over 99% normal lactone in a whole cell biocatalysis by the triple variant (Supporting Information Figure 3C).

A total of 2 mg of the purified enzymes fully converted 2 mM substrate after less than 30 min in the case of the wild type and around 150 h in the case of the triple variant (Supporting Information Figure 3B). Although these results show a decreased activity of the triple variant compared to the wild type, they also demonstrate the high stability of this enzyme. Even after several days, the enzyme still continuously converts the substrate. In general, the high stability of $CHMO_{Arthro}$ is a

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major advantage of this enzyme, making it worth being studied in more detail. When using purified enzyme, not only the activity but also the amount of normal lactone was decreased, and varying ratios of normal to abnormal lactone were obtained. This observation was made for all mutants with the F299A mutation. We suppose that F299 is relevant for FAD coordination, and by mutating this residue, FAD is lost while purifying the enzyme, which already becomes evident by the missing yellow color of the purified fractions. Thus, we tried to circumvent the loss of cofactor by adding FAD in excess to all buffers used for purification. However, still only 90−94% of normal lactone was produced by the triple mutant. We assume that FAD is bound slightly different after reconstitution, and this might already lead to a different substrate positioning toward the peroxy group bound to the FAD, giving rise to the possibility that other bonds are in an antiperiplanar orientation. The change in the binding of FAD and possibly also in the enzyme conformation during purification of the variants might not be the reason for only a changed regioselectivity but also for the decreased activity of the purified enzyme variants. It should also be noted that experiments at different oxygen concentrations did not reveal any change in the regioselectivity.

In summary, we established the first example for a complete switch in the regioselectivity of Baeyer−Villiger monooxygenases as exemplified for $CHMO_{Arthro}$ toward (+)-*trans*-dihydrocarvone by rational design by a stepwise protein engineering strategy and for $CHMO_{Acineto}$ in order to demonstrate the value of our computational design. To achieve this, it was necessary to fully understand the mechanism and structural changes during this oxidation reaction. However, the first key mutation F299A could not have been found by rational design, and it is unclear how this single mutation contributes to the change in regioselectivity. By building homology models of the wild type enzyme and the single and double mutants using the $CHMO_{Rhodo}$ *tight* structure as a template and assuming that the directionality of $CHMO_{Arthro}$ and $CHMO_{Rhodo}$ comply with each other, it was then possible to determine the substrate positioning for the formation of normal and abnormal lactone, respectively. This substrate positioning coincides with the one as predicted by Yachnin et al. from which they were able to predict the right lactone product by two different BVMOs.[23] When looking at the best docking result of the F299A/F330A/F485A variant, it becomes evident that this changed substrate positioning is only possible in conjunction with the F299A mutation. All F299A/F330A derived triple variants showed a higher ratio of normal lactone than the double mutant, which confirms the value of the $CHMO_{Rhodo}$ *tight* structure for predicting enantio- and regioselectivity of CHMOs exhibiting a high homology to $CHMO_{Rhodo}$. The best enzyme variant F299A/F330A/F485A converted (+)-*trans*-dihydrocarvone almost exclusively to the normal lactone. We believe that our work contributes to the understanding on how regioselectivity is controlled, as it highlights the impact of steric effects on the substrate positioning in the active site. The general assumption is that the more nucleophilic and higher substituted carbon center will migrate during the reorganization of the Criegee intermediate due to better stabilization. However, as the migrating bond has to be in an antiperiplanar position to the peroxy bond in the Criegee intermediate, this rule can only be applied when steric effects in the active site of the enzyme allow the positioning of the substrate in this way. In the case of (+)-*trans*-dihydrocarvone, the active sites of wild type $CHMO_{Arthro}$ and $CHMO_{Acineto}$ are built in a way that the substrate cannot be bound in order to yield the normal lactone. By creating sufficient space, the substrate can bind in an inverted position, and only the normal product will be produced, as this follows the rule of migration of the higher substituted carbon center. The design process of the variant shows on one hand that in order to change the regioselectivity of a BVMO it is essential to understand the mechanism of the reaction and to know the directionality of the enzyme. On the other hand it became evident that rational design alone cannot always pave the way to the desired enzyme property. Sometimes it is necessary to investigate a lot of variants in order to find the right starting point for rational protein design.

METHODS

See the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00723.

Information on the first generation mutants. Figures supporting the general characterization of the $CHMO_{Arthro}$ wild type and the time course measurements (PDF)

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Notes

The authors declare no competing financial interest.

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