Manipulating the stereoselectivity of the thermostable Baeyer–Villiger monooxygenase TmCHMO by directed evolution†

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Baeyer–Villiger monooxygenases (BVMOs) and evolved mutants have been shown to be excellent biocatalysts in many stereoselective Baeyer–Villiger transformations, but industrial applications are rare which is partly due to the insufficient thermostability of BVMOs under operating conditions. In the present study, the substrate scope of the recently discovered thermally stable BVMO, TmCHMO from Thermocrispum mucipare, was studied. This revealed that the wild-type (WT) enzyme catalyzes the oxidation of a variety of structurally different ketones with notable activity and enantioselectivity, including the desymmetrization of 4-methylcyclohexanone (99% ee, S). In order to induce the reversal of enantioselectivity of this reaction as well as the transformations of other substrates, directed evolution based on iterative saturation mutagenesis (ISM) was applied, leading to (R)-selectivity (94% ee) without affecting the thermostability of the biocatalyst.

Introduction

The Baeyer–Villiger oxidation of ketones or aldehydes is a valuable transformation in synthetic organic chemistry in which a single oxygen atom is inserted into the respective C–C bond adjacent to a carbonyl moiety with formation of esters or lactones.1 Many different reagents such as peracids or hydroperoxides have been used as stoichiometric oxidants, sometimes in the presence of chiral transition metal catalysts2 or organocatalysts3 for obtaining enantioselective products. The C–C activating process can also be catalyzed by Baeyer–Villiger monooxygenases (BVMOs),4 which require only dioxygen from air, thereby avoiding the use of potentially explosive reagents. Mechanistically, O2 reacts with the reduced form of the enzyme-bound flavin (FAD), brought into this redox state by NADPH, with formation of an intermediate hydroperoxide. This intermediate in the anionic form adds to the carbonyl function leading to the so-called Criegee intermediate. In the final step, one of the alkyl or aryl groups next to the original carbonyl function migrates. The product is then released, after which NADP+ is also released. Being NADPH-dependent, BVMOs require a cofactor regeneration system such as glucose dehydrogenase or a whole-cell system.

By using BVMOs from different sources and numerous structurally different ketones as non-native substrates, high stereoselectivity has been observed numerous times.4 Moreover, directed evolution5 can be applied in those cases in which stereoselectivity proves to be moderate to poor, or if inversion of the enantioselectivity is required.6 For example, cyclohexanone monoxygenase from Acinetobacter calcoaceticus NCIMB 9871 (AcCHMO) has been subjected to protein engineering a number of times.7 Nevertheless, a major obstacle for wide industrial applications appears to persist, namely the lack of robustness of most BVMOs under operating conditions.8 The discovery of the thermostable phenylacetone monoxygenase from Thermobifida fusca (PAMO) set a milestone in BVMO research, although the respective substrate scope proved to be limited.9 Protein engineering of PAMO, especially by means of directed evolution, was applied several times with formation of variants that show moderate to excellent activity and stereoselectivity, depending upon the nature of the substrate.10 A different approach would be to apply protein engineering for achieving significant thermostability of AcCHMO without a
Results and discussion

As the model reaction for assessing the enantioselectivity of TmCHMO, we chose the BV-oxidation of 4-methyl-cyclohexanone (1a) with formation of (R)- and (S)-2a (Scheme 1). We discovered that in whole-cell reactions, WT TmCHMO shows excellent enantioselectivity and conversion in favour of (S)-2a (99% ee; conversion 100%/24 h). Thus, in this particular transformation, the result is comparable to the use of AcCHMO, the most prominent prototype enzyme of this family, which is likewise (S)-selective (>98% ee). However, TmCHMO has the advantage that it is more thermally stable, the melting temperature (Tm) of the wildtype being 49.8 °C as determined here and reported earlier, compared to AcCHMO (Tm = 37 °C).

We then set out to invert stereoselectivity by protein engineering. Lactone (R)-2a is a chiral compound of particular interest because it is a precursor of (R)-β-methyl-substituted adipic acid, which in turn is an important intermediate in the synthesis of an effective inhibitor of acetylcholinesterase (AChE) as shown in Scheme 2. In earlier work, a mutant of cyclopentanone monoxygenase from Comamonas sp. was shown to be (R)-selective (96% ee) in this reaction, but this BVMO, as in the case of AcCHMO, lacks sufficient thermostability and robustness under operating conditions to be of practical significance. A PAMO variant was also shown to be (R)-selective (98% ee), but activity proved to be poor.

We started our investigations with directed evolution based on saturation mutagenesis at sites surrounding the binding pocket (CAST-sites; Combinatorial Active-site Saturation Test) and iterative saturation mutagenesis (ISM). Utilizing the X-ray structure of TmCHMO (pdb accession code 5M10), we docked the substrate 1a in the active site. Subsequently, 11 amino acids within 5 Å from the docked substrate were chosen for saturation mutagenesis (L145, L146, F248, F279, R329, F434, T435, N436, L437, W492 and F507) (Fig. 1). These positions were subjected individually to NNK-based randomization in which all 20 canonical amino acids are used as building blocks, requiring in each case the screening of about 96 transformants for 95% library coverage (one microtiter plate).

Such exploratory experiments are fast and deliver valuable information for subsequent mutagenesis steps. In the mini-libraries generated by saturation mutagenesis at positions 146, 434, 435, 437 and 507, several mutants were discovered showing decreased (S)-selectivity, namely L146E, F434I, T435F, T435Y, T435W, L437G, L437T, L437A and F507W, thereby pointing the way towards reversal of enantioselectivity. The libraries created at the other six positions failed to harbor any positive variants (Table S1†). At this stage we explored two mutagenesis strategies in parallel. In one approach, saturation mutagenesis at a relatively large 5-residue randomization site defined by the above hot spots was performed. This time, appropriately reduced amino acid alphabets, individually designed for each of the five residues were used. The respective design was based on the information that the initial positive mutants provide (Table S2†). Accordingly, 146E/L, 434I/F, 435F/Y/W/T, 437T/A/G/L and 507W/F, including the amino acid alphabets encoding wild-type and positive mutations at individual positions, were chosen for creating a 5-residue saturation mutagenesis library.

Scheme 1 Baeyer–Villiger oxidation of 4-methylcyclohexanone (1a) catalyzed by TmCHMO.

Scheme 2 Synthesis of (R)-β-methyl-substituted adipic acid, the precursor of an AChE-inhibitor.
A total of only 384 transformants were screened for 95% library coverage, and three (R)-selective variants were indeed identified (Table S3†) showing moderately reversed enantioselectivity (50%–66% ee).

According to the second strategy, the best hit identified in the exploratory NNK-based mini-libraries, mutant L437A, was chosen as a template for performing iterative saturation mutagenesis (ISM) at the remaining four hot spot residues (L146, F434, T435 and F507), which were grouped into two randomization sites: A (F434, T435) and B (L146, F507). Thereafter, several ISM pathways were explored using the respective best variants as templates and NDT codon degeneracy encoding only 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, and Gly) (Fig. 2). Two excellent (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee) and LGY3-4-E5 (F434I/T435L/L437A/F507V) (91% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage, and three (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage, and three (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage, and three (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage, and three (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage, and three (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage, and three (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage, and three (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage, and three (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage, and three (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage, and three (R)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-D11 (F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†).
Table 1  Kinetic and thermostability data of WT TmCHMO, and mutants LGY3-4-D11 and LGY3-4-E5. 4-Methylcyclohexanone (1a) was used as substrate for the kinetic analysis

<table>
<thead>
<tr>
<th>Entry</th>
<th>Mutations</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ ($\mu M$)</th>
<th>$K_i$ (mM)</th>
<th>$k_{unc}$ ($s^{-1}$)</th>
<th>$T_m$ (°C)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>1.93 ± 0.03</td>
<td>&lt; 1</td>
<td>13 ± 3</td>
<td>0.22 ± 0.08</td>
<td>49.8</td>
</tr>
<tr>
<td>LGY3-4-D11</td>
<td>L146F/F434I/T435L/L437A/F507C</td>
<td>0.2$^a$</td>
<td>n.d.$^a$</td>
<td>n.d.$^a$</td>
<td>0.16 ± 0.01</td>
<td>53.0</td>
</tr>
<tr>
<td>LGY3-4-E5</td>
<td>F434I/T435L/L437A/F507V</td>
<td>1.23 ± 0.09</td>
<td>5500 ± 750</td>
<td>47 ± 8</td>
<td>0.053 ± 0.002</td>
<td>51.5</td>
</tr>
</tbody>
</table>

$^a$ n.d. = not determined, due to unreliable fitting only a maximal observed rate is given. All experiments were done in triplicates. $^b$ The standard deviation (triplicates) was <0.5 °C.

Table 2  Substrate scope of WT TmCHMO and variants LGY3-4-D11 and LGY3-4-E5

<table>
<thead>
<tr>
<th>Substrate</th>
<th>WT Conversion</th>
<th>ee [%]</th>
<th>LGY3-4-D11 Conversion</th>
<th>ee [%]</th>
<th>LGY3-4-E5 Conversion</th>
<th>ee [%]</th>
</tr>
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<tbody>
<tr>
<td>1a</td>
<td>+++</td>
<td>99(S)</td>
<td>+++</td>
<td>94(R)</td>
<td>+++</td>
<td>91(R)</td>
</tr>
<tr>
<td>1b</td>
<td>+++</td>
<td>18(R)</td>
<td>+++</td>
<td>99(R)</td>
<td>+++</td>
<td>99(R)</td>
</tr>
<tr>
<td>1c</td>
<td>+++</td>
<td>93(S)</td>
<td>n. c.</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
</tr>
<tr>
<td>1d</td>
<td>++</td>
<td>88(−)</td>
<td>+</td>
<td>16(−)</td>
<td>+</td>
<td>30(+)</td>
</tr>
<tr>
<td>3</td>
<td>++</td>
<td>99(4S,6R)</td>
<td>++</td>
<td>99(4S,6R)</td>
<td>+++</td>
<td>99(4S,6R)</td>
</tr>
<tr>
<td>5a</td>
<td>+++</td>
<td>49(R)</td>
<td>+++</td>
<td>99(R)</td>
<td>+++</td>
<td>98(R)</td>
</tr>
<tr>
<td>5b</td>
<td>+++</td>
<td>93(S)</td>
<td>++</td>
<td>94(R)</td>
<td>+++</td>
<td>95(R)</td>
</tr>
<tr>
<td>7</td>
<td>+++</td>
<td>N: ABN = 50:50</td>
<td>+++</td>
<td>55:45</td>
<td>+++</td>
<td>50:50</td>
</tr>
<tr>
<td>9</td>
<td>+++</td>
<td>ee% &gt;99(−), &gt;99(−)</td>
<td>+++</td>
<td>83(−), 99(−)</td>
<td>+++</td>
<td>98(−), 99(−)</td>
</tr>
<tr>
<td>11</td>
<td>+++</td>
<td>P: D = 45:55</td>
<td>ee% 99(−), 99(−)</td>
<td>+++</td>
<td>35:65</td>
<td>30:70</td>
</tr>
<tr>
<td>13</td>
<td>++</td>
<td>97(R)</td>
<td>+</td>
<td>75(R)</td>
<td>+</td>
<td>96(R)</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>98(R)</td>
<td>+</td>
<td>66(R)</td>
<td>+</td>
<td>58(R)</td>
</tr>
</tbody>
</table>

Conversion: +++ >80%, ++ 50–80%, + <50%; P: proximal, D: distal; N: normal, ABN: abnormal.; n.d. not determined, n.c. not converted.

Scheme 3  Baeyer–Villiger oxidation of a series of structurally diverse substrates catalyzed by TmCHMO.
which is (S)-selective (95% ee) (Scheme 3 and Table 2). This reversal is a synthetically significant result because the standard BVMO, AcCHMO, is (S)-selective (81% ee), while the (R)-product constitutes a chiral precursor in the synthesis of the therapeutically useful γ-aminobutyric acid receptor (GABA<sub>A</sub>) agonist (R)-baclofen. As shown in Table 2, all three enzymes convert substrate 7 with a fast reaction rate and almost the same ratio of normal lactone to abnormal lactone (N : ABN %) and high ee value, which is similar for AcCHMO [N : ABN = 51:49, 95(−), 95(−)]. The expected “normal” lactone can be used as an intermediate in the synthesis of the Corey lactone, a versatile building block in the synthesis of prostaglandins. Furthermore, the “abnormal” lactone was used as a starting material in the chemoenzymatic total synthesis of various brown algae pheromones. The conversion results of 3-methyl-cyclohexanone (11) indicate that the mutations improve the tendency of the enzyme to produce more of the distal product. Protein engineering of other BVMOs resulting in a change of the normal/abnormal lactone ratio has been reported earlier. However, the introduced mutations have little effect in reactions of compound 13 and a negative effect in the reaction of 15 (Scheme 3 and Table 2).

**Conclusions**

This study shows that the recently discovered thermally stable Baeyer–Villiger monoxygenase TmCHMO, and the two variants LGY3-4-D11 and LGY3-4-E5, are excellent biocatalysts in the asymmetric transformation of a variety of structurally different ketones. The two mutants were generated by directed evolution utilizing a special version of iterative saturation mutagenesis (ISM). As demonstrated by the determination of the T<sub>m</sub> values, the thermostability of the two TmCHMO mutants is retained relative to WT. In some cases the high enantioselectivity in desymmetrization reactions is similar to those reported for the prototype CHMO, AcCHMO, but this well-known BVMO is only moderately stable. Moreover, reversal of enantioselectivity as reported herein allows ready access to several chiral compounds of particular synthetic value. The overall results of this study show that TmCHMO is an attractive scaffold for future mutagenesis. It will be interesting to see how TmCHMO and the two generated variants perform in BV-reactions of other ketones, and as biocatalysts in enantioselective sulfoxidations. Further ISM studies starting from WT or the two mutants may also be a rewarding task in future work. Finally, a complete theoretical study, hopefully unveiling the molecular basis of stereoselectivity, would not only be of fundamental interest, it could also guide future directed evolution of this promising BVMO.

**Conflicts of interest**

There are no conflicts of interest to declare.

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**Notes and references**


