Asymmetric Catalysis

Directed Evolution as a Method To Create Enantioselective Cyclohexanone Monooxygenases for Catalysis in Baeyer–Villiger Reactions**

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Dedicated to Professor Jean-Marie Lehn on the occasion of his 65th birthday

The Baeyer–Villiger (BV) reaction of ketones with hydroperoxides affords the corresponding esters or lactones.[1] The reaction is accelerated by acids, bases, or metal complexes, and enantioselective catalysis is possible in certain cases.[1,2] Alternatively, flavin-dependent enzymes such as cyclohexanone monooxygenases (CHMOs) can be used as biocatalysts in asymmetric BV reactions.[3,4] In this enzymatic process dioxygen (air) reacts with the enzyme-bound flavin (FAD) to form an intermediate hydroperoxide, which in the deprotonated form initiates the BV reaction by transferring one oxygen atom originating from O_2 to the substrate (ketone), the other being ultimately reduced to water.[3–6] This means that the flavin co-factor needs to be recycled by reduction, the second co-factor NADPH taking over this function. Consequently, when employing the isolated CHMOs, NADPH has to be recycled. Although NADPH recycling systems are well known,[3] it is currently more convenient to use whole cells expressing CHMO. This process has been employed successfully for a number of enantioselective BV reactions, such as kinetic resolution and desymmetrization of prochiral substrates.[4,6] Of course, many ketones fail to react with accept-

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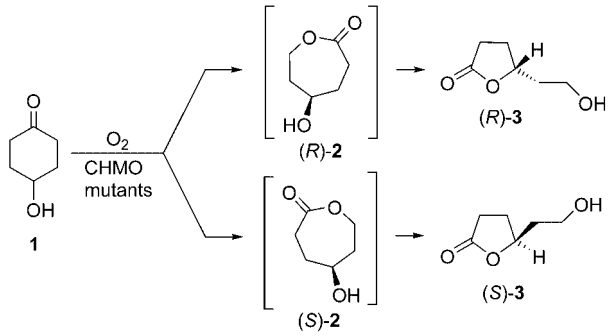
able levels of enantioselectivity. Here we show that the methods of directed evolution[7] encompassing repetitive cycles of gene mutagenesis, expression, and screening are ideally suited to solve this problem. Previously we demonstrated that this new approach to asymmetric catalysis can be used to evolve enantioselective lipases[8] and epoxide hydrolases,[9] and that the lessons learned from directed evolution following appropriate theoretical analysis provide valuable structural and mechanistic insights.[10]

For our study we chose the desymmetrization of 4-hydroxycyclohexanone (**1**) as the model reaction and the CHMO from *Acinetobacter* sp. NCIMB 9871 (EC 1.14.13.22)[5b] as the "Baeyer–Villigerase", specifically whole-cell preparations of *E. coli* expressing this recombinant enzyme.[11] The BV reaction of ketone **1** leads to the lactone (*R*)/(*S*)-**2**, which spontaneously rearranges to (*R*)/(*S*)-**3** without change in stereochemistry. The primary process provides an enantiomeric excess (9% *ee*; wild-type) of (*R*)-**3**, which is opposite to the usual *S* selectivity that is generally observed for 4-substituted cyclohexanone derivatives using this enzyme as a catalyst.[3–6]



As in our previous studies on directed evolution of enantioselective enzymes,[8,9] we began exploring protein sequence space by applying the error-prone polymerase chain reaction (epPCR) as the mutagenesis method.[7,12] Accordingly, several libraries of mutant CHMO genes were produced under different epPCR conditions. In doing so the $MgCl_2$ concentration and other parameters were varied which led to different mutagenesis rates.[12] After insertion of the genes into the *E. coli* host,[11] plating on agar plates, and harvesting the bacterial colonies with the help of a colony picker, the whole cells were placed in the deep wells of 96-format microtiter plates and used as biocatalysts in the model reaction. The product was extracted with ethyl acetate by using robotic equipment followed by analysis by using an adapted form of our previously described medium-throughput GC-based screening system,[13] which allows about 800 *ee*-determinations per day.

Upon screening a total of 10000 mutants, at least two dozen hits showed enhanced *R* selectivity, while about one dozen mutants displayed reversal of enantioselectivity in favor of (*S*)-**3**. A total of eight mutants were sequenced (Table 1), in which between one and three amino acids were exchanged.

Table 1: Altered CHMO mutants identified in the first round of epPCR (reaction time: 24 h; 23–25 °C; >95% conversion).

Mutant	Amino acid exchanges	Favored enantiomer of **3**	*ee* [%]
wild-type	–	*R*	9
1-C2-B7	F432Y, K500R	*R*	34
1-F1-F5	L143F	*R*	40
1-E12-B5	F432I	*R*	49
1-H7-F4	L426P, A541V	*R*	54
1-H3-C9	L220Q, P428S, T433A	*S*	18
1-F4-B9	D41N, F505Y	*S*	46
1-K6-G2	K78E, F432S	*S*	78
1-K2-F5	F432S	*S*	79

Evolutionary optimization of *R* selectivity was then attempted by using the genes of some of the *R*-selective mutants as starting points for a second round of epPCR. In each case about 1600 mutants were screened. Although a systematic search was not strived for at this stage, this strategy turned out to be successful. In the case of 1-F1-F5 (40% *ee*), which is characterized by a single mutation L143F, a second round of epPCR led to a markedly improved mutant (2-D19-E6). The latter catalyzes the BV reaction with an *ee* value of 90% in favor of (*R*)-**3**. Sequencing revealed that three new amino acid exchange events have occurred (E292G, L435Q, T464A) in addition to the already existing L143F mutation.

Previous research has shown that the mechanism of the CHMO-catalyzed BV reaction is similar to that of the classical synthetic process under basic conditions[1] in that the hydroperoxide anion adds nucleophilically to the carbonyl moiety followed by σ-bond migration.[3–6] The fundamental difference is that in the enzymatic reaction the process occurs in the protein environment (Figure 1). As stereoselective σ-bond migration proceeds, a geometric change can be anticipated that accompanies ring expansion. One factor that will

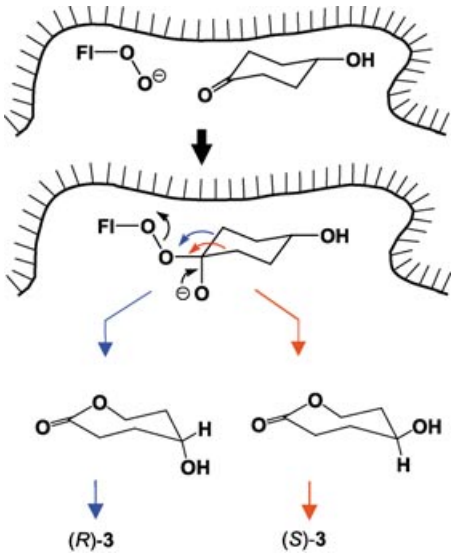
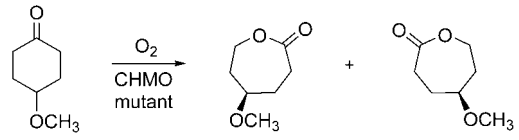


Figure 1. Scheme for the CHMO-catalyzed Baeyer–Villiger reaction of 4-hydroxycyclohexanone (Fl = flavin). Migration of the enantiotopic σ-bond indicated by the blue arrow leads to (*R*)-**3**, whereas migration of the other σ-bond (red arrow) initiates the formation of (*S*)-**3**.

influence enantioselectivity in the present case is the fact that the two ring expansion modes place the 4-hydroxy moiety with its potential H-bond donor capability in two different protein environments prior to rearrangement. Since crystallographic data on the wild-type CHMO is not available at this time, we refrain from more detailed speculations regarding models that have been proposed in other CHMO-catalyzed BV reactions.[14] We do not yet have kinetic data concerning the catalytic activity of the various mutants, although all of them induce >95% conversion under the standard conditions (24 h, 23–25 °C).

Directed evolution of an enantioselective enzyme for a given substrate does not necessarily mean narrow substrate acceptance.[8] To study this important aspect, the catalytic performance of some of the evolved mutants was examined in the BV reaction of various 4-substituted cyclohexanone derivatives that lead to the corresponding seven-membered lactones. We regarded 4-methoxycyclohexanone (**4**) to be of particular interest, because the methoxy group can function as a hydrogen acceptor, but not as a hydrogen donor.



4 (*R*)-**5** (*S*)-**5**

The wild-type CHMO from *Acinetobacter* sp. NCIMB 9871 catalyzes the BV reaction of ketone **4** with *S* selectivity (78% *ee*), similar to certain 4-alkyl derivatives of cyclohexanone.[11] This means that all of these substrates show the opposite enantioselectivity relative to that of the hydroxy substrate **1**. Mutant 2-D19-E6, which had previously been shown to be highly *R* selective in the reaction of hydroxy ketone **1** (90% *ee*), leads to a low degree of enantioselectivity in the reaction of the methoxy ketone **4** (25% *ee* in favor of (*R*)-**5**). In contrast, the most *S*-selective mutant (1-K2-F5) in the reaction of **1** (Table 1) shows essentially complete enantioselectivity when catalyzing the BV reaction of **4** (98.6% *ee* in favor of (*S*)-**5**). These observations suggest that this mutant has undergone an amino acid substitution as a consequence of which an additional hydrogen bond is formed to the methoxy moiety in the transition state of the reaction of **4** and/or shape complementarity is allowed. Indeed, 1-K2-F5 is characterized by a single amino acid substitution at position 432, serine replacing phenylalanine (F432S).

We then studied the catalytic performance of mutant 1-K2-F5 in the BV reaction of other 4-substituted cyclohexanone derivatives, such as the methyl, ethyl, chloro, bromo, and iodo compounds that are known to be converted by the wild-type CHMO with 95–99% *ee*.[15] In no case did we observe any substantial reduction in enantioselectivity. Thus, this mutant accepts a relatively wide range of substrates and catalyzes conversion with 95–99% *ee*.

Since amino acid number 432 seems to be a particularly sensitive "hot spot", hosting a mutation in four different CHMO variants (Table 1), we performed saturation mutagenesis at this position. We expect all of the theoretically possible 20 mutants to be present in the 300–400 bacterial colonies studied. This mini-library was then screened in the BV reaction of **1**. Several mutants displaying altered enantioselectivity were identified and sequenced, including two *R*-selective variants, 1-D10 (F432Y; 17% *ee*) and 4-D11 (F432P; 72% *ee*), and two *S*-selective counterparts, 1-C8(F432G; 17% *ee*) and 1-D5 (F432S; 79% *ee*). 1-D5 is identical to 1-K2-F5 that had been created and identified in the first cycle of epPCR. As shown in the following paper in this issue, this mutant also catalyzes the enantioselective oxidation of prochiral thioethers.[16]

In summary, we have demonstrated that the methods of directed evolution can be applied successfully to the creation of enantioselective cyclohexanone monooxygenases as catalysts in Baeyer–Villiger reactions of several different substrates, for which the enantioselectivity ranges between 90% and 99%.[17] This was accomplished without any knowledge of the three-dimensional structure of the enzyme, which would not be possible in a traditional approach based on "rational design".[3] So far only a small portion of protein sequence space has been explored, the total number of mutants screened being less than 20000. Crystallographic data of the wild-type CHMO and of the enantioselective mutants would be of great interest for a sound theoretical analysis.

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