Asymmetric Catalysis

Directed Evolution of Cyclohexanone Monooxygenases: Enantioselective Biocatalysts for the Oxidation of Prochiral Thioethers**

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In the preceding communication in this issue we reported that the methods of directed evolution can be used to evolve enantioselective mutants of the cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871 (EC 1.14.13.22)[1] as catalysts in the Baeyer–Villiger (BV) reaction of 4-hydroxycyclohexanone and other 4-substituted cyclohexanone derivatives.[2] Since the isolated form of this flavin-dependent enzyme requires co-factor regeneration, we preferred to use whole cells, dioxygen from air serving as the oxidant. In view of the well-known fact that CHMOs can also

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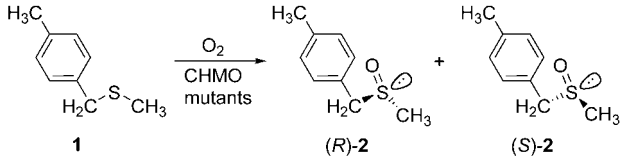
be used as a catalyst in the enantioselective air-oxidation of certain prochiral thioethers with formation of chiral sulfoxides,[1,3] we posed the question whether directed evolution can be applied in the quest to enhance enantioselectivity in "difficult" cases of this reaction type, that is, when the wild-type enzyme results in poor *ee* values. As an example, we chose the oxidation of methyl-*p*-methylbenzyl thioether (**1**) with formation of the chiral sulfoxide **2**, the wild-type CHMO from *Acinetobacter* sp. NCIMB 9871 leading to an *ee* of only 14% in favor of (*R*)-**2**.[3b] It was of practical and theoretical interest to evolve both *S*- and *R*-selective CHMOs, because this allows for enantiodivergence on an optional basis.



As in our previous studies on the directed evolution of enantioselective enzymes,[2,4] we started the exploration of protein sequence space by applying error-prone polymerase reaction (epPCR).[5] However, since the first round of epPCR has no evolutionary character, new mutagenesis experiments were not necessary, that is, we initially used the mutant CHMOs produced previously.[2]

The development of an appropriate *ee* assay was not a trivial task, because previously devised high-throughput screening systems[6] were not easily adaptable in the present case. We finally modified a commercially available HPLC instrument equipped with a sample manager and the appropriate software to handle 96- (or 384-)well microtiter plates. Since enantiomeric separation of (*R*)-**2**/(*S*)-**2** has to be fast and efficient, exploratory experiments were performed by using a variety of different chiral stationary phases, solvents, and conditions. An efficient system turned out to be benzoylated cellulose as the stationary phase with a mixture of *n*-heptane and ethanol as the mobile phase. For rapid analysis short columns 50 mm in length and 4.5 mm in diameter were used. This system allows at least 800 *ee*-determinations per day. Unfortunately, the *E. coli*-based expression system[7] produces small amounts of indole,[8] which leads to an overlap with the HPLC peak of (*S*)-**2**. Although this can be considered in the quantitative evaluation, the *ee* values accessible under these conditions are not precise and were consequently used only to identify hits. These were subsequently analyzed by a similar HPLC setup using a longer column (250 mm) that allows about 40 precise *ee*-determinations per day.

There was no reason to believe that the most enantioselective CHMO mutants evolved in our previous study concerning the BV reaction of cyclohexanone derivatives[2] should also function well as biocatalysts in a completely different reaction type involving a thioether that has no structural similarity with the previously used cyclohexanone-derived substrates. Therefore we did not focus on the original hits,[2] but rather screened the complete 10000-membered library.[2] This led to the discovery of at least 20 mutants having *ee* values in the range 85%–99%, some being *R*- and others being *S*-selective. Five mutants resulting in *ee* values of $>95\%$ were sequenced (Table 1).[3c]

Table 1: Selected mutant CHMOs from *Acinetobacter* sp. NCIMB 9871 for the enantioselective air-oxidation of thioether **1** (24 h; 23–25 °C) using whole cells.

Mutant	Amino acid exchanges	Yield of **2** [%]	Configuration	*ee* [%]	Sulfone **3** as side product [%]
wild-type	–	75	*R*	14.0[a]	<1
1-D10-F6	D384H	75	*R*	98.9	7.9
1-K15-C1	F432S	55	*R*	98.7	20.0
1-C5-H3	K229I, L248P	77	*S*	98.1	5.6
1-H8-A1	Y132C, F246I, V361A, T415A	52	*S*	99.7	26.6
1-J8-C5	F16L, F277S	84	*S*	95.2	5.6

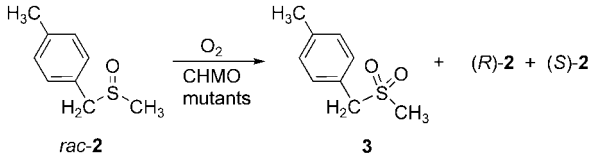
[a] Only 5% *ee* was obtained when the enzyme extract was used.

It can be seen that two mutants (1-D10-F6 and 1-K15-C1) are *R*-selective, while the other three variants (1-C5-H3, 1-H8-A1 and 1-J8-C5) induce the opposite enantioselectivity in favor of (*S*)-**2**. Sequencing studies show that between one and four amino acid exchanges have occurred. In four cases the mutants are different from those that were previously identified as hits in the BV reaction of prochiral cyclohexanone derivatives.[2] In contrast, we were surprised to learn that mutant 1-K15-C1, which leads to 98.7% *ee* in favor of (*R*)-**2**, is characterized by amino acid exchange F432S and is therefore identical to mutant 1-K2-F5 previously identified as a highly enantioselective biocatalyst in the asymmetric BV reaction of a wide range of 4-substituted cyclohexanone derivatives.[2] Thus, one and the same single mutational change at position 432, namely the introduction of serine, leads to a surprisingly versatile biocatalyst. It was identified twice by screening the same 10000-membered library in two different reaction types.

We conclude that directed evolution provides mutant CHMOs that would hardly have been obtained by traditional methods based on rational design and site-specific mutagenesis, especially because the crystal structure of the wild-type is not (yet) available.[1–3] In view of the lack of structural data it is too early to discuss the origin of enantioselectivity of 1-K15-C1 (1-K2-F5) or of the other mutants.

Careful analysis of all products formed under the conditions revealed another notable effect. In all cases small amounts of over-oxidation with formation of achiral methyl-*p*-methylbenzyl sulfone (**3**) were observed (Table 1), which could influence the degree of enantioselectivity either in a positive or a negative manner. This effect is known to occur in CHMO-catalyzed and other microbial oxidations of prochiral thioethers,[1,3,9] although it is usually small due to the low rate of over-oxidation. Depending upon the particular system, the effect can increase or decrease the final measured enantiopurity of the primary product **2**. Similar effects (positive or negative) have been reported in the titanium-catalyzed oxidation of thioethers using the Sharpless/Kagan/Modena

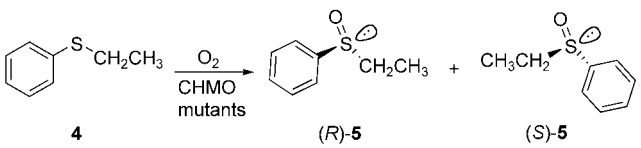
systems.[10] We therefore prepared racemic sulfoxide **2** and performed the kinetic resolution[11] using the *R*- and *S*-selective mutants 1-D10-F6 and 1-H8-A1, respectively. In both cases the usual conditions were applied (24 h; 23–25 °C), each reaction being carried out three times.



In the case of mutant 1-D10-F6 the reaction after 24 h led to 43% conversion to the sulfone **3** with concomitant enrichment of (*R*)-**2** (98.7% *ee*).[12] This means that (*S*)-**2** is consumed preferentially. Thus, in the original desymmetrization of prochiral sulfoxide **1** mutant 1-D10-F6 functions *cooperatively* in two different catalytic reactions, namely in the highly favored formation of (*R*)-**2** and in the enantioselective oxidative destruction of the opposite enantiomer (*S*)-**2**. An analogous effect was observed in the case of mutant 1-H8-A1, which after a 24 h reaction of *rac*-**2** leads to 62% formation of sulfone **3** with concomitant enrichment of (*S*)-**2** (98.9% *ee*). Thus, the process of random mutagenesis/screening leads to the evolution of highly enantioselective biocatalysts for two different oxidative processes, in both cases *R*- and *S*-selectivity being possible on an optional basis. In contrast, the wild-type is not only a poor catalyst in the desymmetrization of **1** (Table 1), but also fails in the oxidative kinetic resolution of *rac*-**2** (after 24 h about 18% sulfone **3** and 6% *ee* in slight favor of (*R*)-**2**).

We then attempted to apply directed evolution to reduce the amount of sulfone formation while maintaining high enantioselectivity in the desymmetrization of thioether **1**. After performing epPCR with the gene encoding mutant 1-H8-A1 and screening a library of 1600 clones for high *S* selectivity *and* low sulfone-formation, mutant 2-K11-F11 was identified. It is characterized by three of the four mutations of the parent mutant (Y132C, V361A, and T415A) and by three new amino acid exchanges Q92R, F246N, and P169 L. This mutant leads to 99.8% *ee* in favor of (*S*)-**2**, the amount of undesired sulfone **3** being almost negligible (< 5%).

Finally, to test whether the best mutant CHMOs evolved for substrate **1** are also efficient biocatalysts in the oxidation of structurally different thioethers, ethylphenyl thioether **4** was used as the substrate. The wild-type CHMO leads to an *ee* of only 47% in favor of (*R*)-**5**.[3b] Mutant 1-J8-C5 results in a pronounced enhancement of *R* selectivity (88% *ee*), whereas mutant 1-C5-H3 catalyzes the complete reversal of enantioselectivity in favor of (*S*)-**5** (98.9% *ee*).



In summary, directed evolution is ideally suited to control the direction and degree of enantioselectivity in the CHMO-catalyzed air-oxidation of prochiral thioethers. The evolved mutants showing highest enantioselectivity for the reaction of a given substrate can also be used as catalysts for other substrates. Finally, the methods of directed evolution not only provide biocatalysts for the enantioselective oxidation of thioethers, but also for the efficient kinetic resolution of racemic sulfoxides. We are currently expanding the range of substrates and reaction types catalyzed by mutant CHMOs.

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[12] We noticed some variation when computing the selectivity factor *E* according to the formula of Sih[11a] even in the conversion range of 25–45 %. Therefore, we refrain from reporting *E* values in this whole-cell system and provide conversion data and *ee* values following reactions under standard conditions (24 h at 23–25 °C).

