

# Self-Sufficient Baeyer–Villiger Monooxygenases: Effective Coenzyme Regeneration for Biooxygenation by Fusion Engineering\*\*

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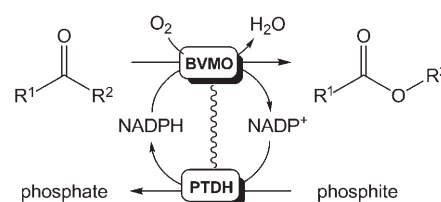
Over the past few years, industrial interest in biocatalysts that perform selective oxidative reactions has increased significantly.<sup>[1]</sup> Baeyer–Villiger monooxygenases (BVMOs) have been identified as a highly versatile class of enzymes for the efficient catalysis of chemo-, regio-, and/or enantioselective oxygenation reactions.<sup>[2]</sup> Although the most prominent transformation catalyzed by these biocatalysts is a chiral variant of the classical Baeyer–Villiger reaction,<sup>[3,4]</sup> the oxygenation of heteroatoms and epoxidation reactions have also been reported.<sup>[5]</sup> Stoichiometric amounts of O<sub>2</sub> and NADPH are required for these reactions. A complication for the large-scale application of these reactions is the high cost of the reduced nicotinamide coenzyme.<sup>[6]</sup> To overcome this problem, several electrochemical and photochemical approaches have been explored.<sup>[7]</sup> However, the efficiency of these approaches is typically poor. Furthermore, it has been shown that BVMOs require NADP<sup>+</sup> for stability and enantioselective catalysis.<sup>[8]</sup>

An efficient and commonly used method for coenzyme regeneration employs whole cells, especially in combination with the recombinant expression of the required biocatalysts.<sup>[9]</sup> This strategy has been implemented in BVMO-mediated biotransformations with wild-type strains<sup>[10]</sup> and has proved particularly successful with recombinant over-expression systems.<sup>[11,12]</sup> The approach avoids laborious enzyme purification steps and exploits the coenzyme regeneration capacity of the host. Although whole cells have been shown to be effective catalysts for Baeyer–Villiger oxidation,<sup>[13]</sup> they also exhibit limitations, such as cellular toxicity,

enzyme inhibition by the substrate/product, degradation of the product, and poor oxygen-transfer rates.<sup>[14]</sup> Coenzyme regeneration by using isolated enzymes has also been studied extensively in the past few years.<sup>[15]</sup> Well-known examples of such NADPH-regenerating enzymes are alcohol dehydrogenase and formate dehydrogenase.<sup>[16]</sup> A phosphite dehydrogenase (PTDH) was also identified as an effective enzyme for coenzyme regeneration.<sup>[17]</sup> The favorable thermodynamic equilibrium constant makes the oxidation of phosphite a nearly irreversible process.<sup>[18]</sup> The exquisite selectivity of PTDH for phosphite also precludes any side reactions, such as those that can occur, for example, when an alcohol dehydrogenase is used. These characteristics make PTDH an ideal candidate for use as a coenzyme regenerating enzyme (CRE) in combination with BVMOs or other NAD(P)H-dependent enzymes.

Herein, we report a novel approach to the combination of the catalytic activity of a redox biocatalyst with concomitant coenzyme recycling in a single fusion protein (Scheme 1). During the last decade, a number of fusion protein tags have been developed. These tags are used intensely in life-science-related research and commercial activities. Although the fusion of proteins is a widely applied strategy in, for example, enzyme purification (e.g. the use of glutathione S transferase (GST) tags)<sup>[19]</sup> and the subcellular visualization of target proteins (e.g. with a green fluorescent protein (GFP) tag),<sup>[20]</sup> this concept is hardly ever encountered in the context of synthetic applications. Only a few isolated examples in the literature provide evidence that the fusion of separate enzymes can result in improved biocatalytic properties.<sup>[21]</sup>

We report herein on the engineering of a number of representative BVMOs that are linked covalently to soluble NADPH-regenerating phosphite dehydrogenase. This construct enables the use of phosphite as a cheap and sacrificial electron donor with whole cells, cell extracts, and purified enzyme. It was our particular goal to design a self-sufficient two-in-one redox biocatalyst that does not require an additional catalytic entity for coenzyme recycling. As model



**Scheme 1.** Coenzyme regeneration by CRE/BVMO fusion enzymes. NADPH is the reduced form of nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>).

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[\*\*] We thank CERC3 and the Austrian Science Fund (FWF project no. I19-B10) for funding, and W. A. van der Donk, University of Illinois, for providing the pRW2 double-mutant template. This study was conducted in association with COST action D25 - Applied Biocatalysis within the working group "Biooxidation".

Supporting information for this article, including experimental details, is available on the WWW under <http://www.angewandte.org> or from the authors.

BVMOs, we selected thermostable phenylacetone monooxygenase (PAMO) from *Thermobifida fusca*,<sup>[22]</sup> well-studied cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp.,<sup>[23]</sup> and cyclopentanone monooxygenase (CPMO) from *Comamonas* sp.<sup>[24]</sup> To overexpress such CRE/BVMO fusion proteins, we constructed expression vectors (pCRE) that result in the expression of BVMOs fused to PTDH by a short linker peptide. We formed two fusion enzymes with PAMO by linking PAMO to the N or C terminus of PTDH, whereas CHMO and CPMO were linked to PTDH at their N terminus. All four bifunctional enzymes showed excellent expression levels when *E. coli* TOP10 was used as the host. The CRE/BVMO enzymes were purified by column chromatography to yield 10–50 mg of pure and soluble CRE/BVMO from 1 L of culture broth, with the exception of CRE–CPMO, which has never been purified successfully from a recombinant host.<sup>[24]</sup>

Steady-state kinetic analysis of the CRE/BVMO enzymes revealed that the fusion of the BVMOs with PTDH hardly affected their respective catalytic properties at all (Table 1). The rates of catalytic activity ( $k_{\text{cat}}$ ) of the BVMO subunits were similar to those observed for the separate enzymes. The only significant effect observed was a decrease in the affinity of PTDH for phosphite (the  $K_{\text{M}}$  value is approximately 13 times higher) when fused to a BVMO. Nonetheless, the observed kinetic parameters should allow efficient conversions.

No inhibition of the activity of either the BVMO or PTDH by the substrate or product of the other subunit was observed. Activity measurements at elevated temperatures showed that the PAMO and PTDH domains have similar thermostabilities compared to the separate isolated enzymes.<sup>[18,22]</sup> Moreover, we determined the NADPH-regeneration efficiency of PAMO–CRE by following the conversion of phenylacetone (PA; 2.5 mM) with time. In the presence of NADP<sup>+</sup> at a concentration of only 5  $\mu\text{M}$ , the bifunctional fusion enzyme converted effectively phenylacetone into benzyl acetate (79% conversion after 3 h). Control experiments in which equal amounts of units of each the individual enzymes PAMO and PTDH were added in place of the fusion enzyme yielded similar results (Table 2). In similar experiments carried out recently with PAMO in combination with an alcohol dehydrogenase in a two-liquid-phase system, lower TOF and total turnover number (TTN) values were found in the presence of 125  $\mu\text{M}$  NADP<sup>+</sup>.<sup>[15b]</sup> Strikingly, even in the presence of equimolar amounts of NADP<sup>+</sup> and PAMO–CRE (0.2  $\mu\text{M}$ ), 13% conversion was observed, with a TTN of 1750 with respect to the regeneration of the coenzyme. Unfortunately, we observed a decrease in catalytic activity with time,

**Table 1:** Kinetic parameters of the bifunctional fusion enzymes.<sup>[a]</sup>

|                                     | PAMO–CRE                   | CRE–PAMO                   | CRE–CHMO                   |
|-------------------------------------|----------------------------|----------------------------|----------------------------|
| $k_{\text{cat}}$ (ketone)           | 1.9 s <sup>−1</sup> (70%)  | 2.9 s <sup>−1</sup> (100%) | 14 s <sup>−1</sup> (100%)  |
| $K_{\text{M}}$ (ketone)             | 52 $\mu\text{M}$ (70%)     | 53 $\mu\text{M}$ (70%)     | 3.5 $\mu\text{M}$ (50%)    |
| $K_{\text{M}}$ (NADPH)              | < 1 $\mu\text{M}$ (100%)   | < 1 $\mu\text{M}$ (100%)   | 4 $\mu\text{M}$ (60%)      |
| $k_{\text{cat}}$ (phosphite)        | 4.3 s <sup>−1</sup> (225%) | 6.5 s <sup>−1</sup> (340%) | 2.6 s <sup>−1</sup> (140%) |
| $K_{\text{M}}$ (phosphite)          | 285 $\mu\text{M}$ (1400%)  | 317 $\mu\text{M}$ (1500%)  | 228 $\mu\text{M}$ (1100%)  |
| $K_{\text{M}}$ (NADP <sup>+</sup> ) | 12 $\mu\text{M}$ (340%)    | 15 $\mu\text{M}$ (430%)    | 12 $\mu\text{M}$ (340%)    |

[a] The relative kinetic parameters of the native enzymes are shown in parentheses.<sup>[18,22,23]</sup>

**Table 2:** Conversion of phenylacetone (PA) by either PAMO–CRE (0.2  $\mu\text{M}$ ) or PAMO (0.13  $\mu\text{M}$ ) in the presence of PTDH upon incubation at 30 °C for 3 h.

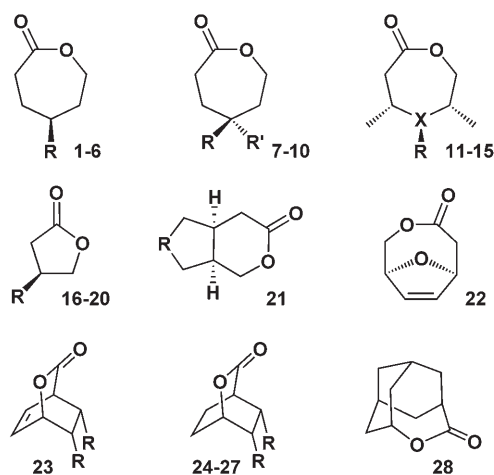
|                             | [PA]<br>[mM] | [NADP <sup>+</sup> ]<br>[ $\mu\text{M}$ ] | TOF <sup>[a]</sup><br>[h <sup>−1</sup> ] | TTN <sup>[b]</sup><br>(PAMO) | TTN <sup>[b]</sup><br>(NADP <sup>+</sup> ) |
|-----------------------------|--------------|-------------------------------------------|------------------------------------------|------------------------------|--------------------------------------------|
| PAMO–CRE                    | 2.5          | 5                                         | $3.3 \times 10^3$                        | $9.9 \times 10^3$            | 395                                        |
| PAMO and PTDH               | 2.5          | 5                                         | $4.5 \times 10^3$                        | $1.4 \times 10^4$            | 360                                        |
| PAMO and ADH <sup>[c]</sup> | 7.5          | 125                                       | 98                                       | $4.7 \times 10^3$            | 12                                         |

[a] Turnover frequency, as described in Ref. [25]. [b] Total turnover number with respect to the biocatalyst or the coenzyme, as described in Ref. [25]. [c] Adapted from Ref. [15b].

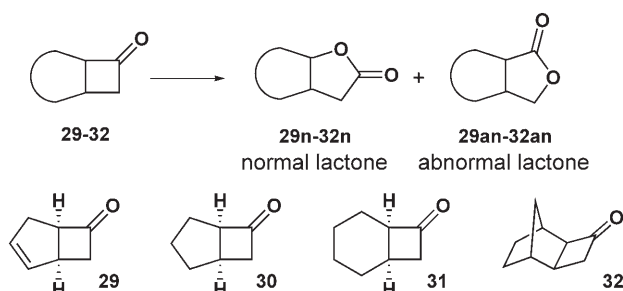
probably as a result of the instability of the enzyme at low concentrations of NADP<sup>+</sup>.

We also studied the applicability of these two-in-one biocatalysts in whole cells and in crude cell extracts by carrying out (enantioselective) transformations with CRE–CHMO and CRE–CPMO. Initial screening experiments to investigate any effect on stereoselectivity of the additional PTDH domain within the bifunctional fusion protein were carried out according to a previously established protocol based on miniscale whole-cell biotransformations.<sup>[26]</sup> We examined the desymmetrization of prochiral substrates, which enables control over up to six stereogenic centers in a single step (Scheme 2), as well as the regiodivergent bio-oxidation of fused racemic cyclobutanones (Scheme 3), one of the most remarkable transformations promoted by BVMOs that oxidize cycloketones (see the Supporting Information).

We observed essentially no adverse influence from the fusion of the two catalytic entities. In the majority of cases, the efficiency of the biotransformation remained similar to that of the equivalent transformation under the catalysis of the original unfused enzymes (as indicated by comparable conversions within standardized reaction times; see the Supporting Information). The stereoselectivity of the fused biocatalysts was also largely comparable to that of the individual enzymes, and in some cases (the formation of **21** with CRE–CHMO and **24** with CRE–CPMO) the product lactones were formed with even better enantioselectivities in the presence of the fusion enzymes. Only in very few cases



**Scheme 2.** Types of lactone products obtained from stereoselective desymmetrization reactions with CRE/BVMO fusion enzymes.



**Scheme 3.** Regiodivergent CRE/BVMO-mediated oxidation of fused bicyclic ketones.

was erosion of optical purity observed in the formation of the lactones (e.g. **12** with both CRE-CHMO and CRE-CPMO); however, the absolute configuration of the products remained unchanged. A more pronounced effect was found with the series of prochiral cyclobutanones. We attribute this to the largely different conformational energies on this ring system compared to structurally better defined cyclopentanones and -hexanones, as recently outlined.<sup>[27]</sup> Remarkably, we observed two cases in which the substrate range of the original BVMO was extended as a consequence of the fusion process (the formation of **10** with CRE-CHMO and **28** with CRE-CPMO). Although in both cases the catalytic efficiency was limited, recently described strategies could be applied to further improve performance.<sup>[28]</sup>

The behavior of the original biocatalysts was also very similar to that of the fused CRE/BVMO enzymes for the regiodivergent oxidation of fused cyclobutanones (see the Supporting Information). Whereas CRE-CHMO yielded equimolar amounts of the “normal” and “abnormal” lactones with high *ee* values in most cases, CRE-CPMO produced predominantly “normal” lactones, but with low stereoselectivity.

With respect to facile and rapid application of these new and self-sufficient CRE/BVMOs in stereoselective synthesis, we prepared a crude cell extract (CE) from CRE-CHMO producing recombinant *E. coli*. The optimum concentrations of both phosphite and 4-methylcyclohexanone as a model substrate were determined (see the Supporting Information), and the fusion biocatalyst as CE was demonstrated to completely convert the ketone (5 mM) into the chiral lactone. A particularly interesting aspect in the utilization of CRE/BVMOs as CE is the fact that the preparative-scale biotransformations outlined above were performed without addition of NADP<sup>+</sup>, taking advantage of the coenzyme present in *E. coli* cells ( $\approx 200 \mu\text{M}$ ).<sup>[29]</sup> Our observation that a relatively low concentration of NADP<sup>+</sup> is sufficient for catalysis suggested that the amount of the nicotinamide coenzyme liberated upon cell breakage should indeed enable effective conversion with cell extracts containing overexpressed CRE/BVMO (cell-pellet volume/incubation volume = 1:2; total protein concentration  $\approx 20 \text{ mg mL}^{-1}$ ). By using a cell extract we also confirmed that the unsaturated bicycloketone precursor to **23** is a substrate for CRE-CHMO. (This biooxidation was reported previously with isolated CHMO;<sup>[30a]</sup> however, when we tried to repeat the experiment by using a recombinant whole-cell strain, only starting material was recovered.<sup>[30b]</sup>) This result underscores a major advantage of

employing bifunctional fusion proteins: Our CRE/BVMO concept in combination with the simple application protocol as a cell extract offers a facile method to establish substrate-acceptance profiles for enzymes with a minimum of effort in terms of protein purification and maximum simplicity with respect to coenzyme regeneration.

In conclusion, we have created a self-sufficient redox biocatalyst by fusing two independent enzymes to form a new bifunctional biocatalyst. The present study demonstrated the feasibility of this novel concept for coenzyme regeneration with three distinct Baeyer–Villiger monooxygenases and a phosphite dehydrogenase for orthogonal coenzyme recycling. The fused BVMOs are complementary in their substrate profiles (PAMO accepts aromatic ketones, CHMO and CPMO accept aliphatic cycloketones) and their stereoselectivity (CHMO and CPMO produce the opposite lactone enantiomers in a large variety of examples). The three monooxygenases are sufficiently different in sequence and in terms of their phylogenetic relationships to suggest the general applicability of this coenzyme regeneration concept, at least among the family of BVMOs. Considering the diverse reactivity of novel members of this family,<sup>[31]</sup> our study may contribute to the further proliferation of this highly interesting biotransformation platform. Presently, we are conducting additional studies to further optimize the efficiency of these newly developed self-sufficient BVMOs for ultimate application in the large-scale production of chiral intermediates for the synthesis of bioactive compounds.

Received: October 8, 2007

Published online: January 25, 2008

**Keywords:** asymmetric catalysis · Baeyer–Villiger reaction · biotransformations · coenzyme regeneration · fusion enzymes

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