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Research review paper

Hot spots for the protein engineering of Baeyer-Villiger monooxygenases

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ABSTRACT

Baeyer-Villiger monooxygenases (BVMOs) are versatile biocatalysts for the conversion of ketones to lactones or esters while also being able to efficiently oxidize sulfides to sulfoxides. However, there are limitations for the application of BVMOs in synthesis. In this review we provide an overview of the protein engineering studies aiming at optimizing different properties of BVMOs. We describe hot spots in the active sites of certain BVMOs that have been successfully targeted for changing the substrate scope, as well as the possibility to influence this property by allosteric effects. The identified hot spots in the active sites for controlling enantio- and regioselectivity are shown to be transferable to other BVMOs and we describe concepts to influence heteroatom oxidation, improve protein stability and change the cofactor dependency of BVMOs. Summarizing all these different studies enabled the identification of BVMO- or property-dependent as well as universal hot spots.

1. Introduction

The classical reaction catalyzed by Baeyer-Villiger monooxygenases (BVMOs) is the enzymatic insertion of an oxygen atom next to the keto-function of the substrate (Scheme 1a). Additionally, many BVMOs are able to oxygenate heteroatoms like sulfur, nitrogen, phosphorus, boron or selenium (Balke et al. 2012; Branchaud and Walsh 1985; de Gonzalo et al. 2010). Compared to the chemically catalyzed reaction first published by Baeyer and Villiger (1899), where peracids are needed as oxidation agents, the enzymatic Baeyer-Villiger oxidation (BVO) uses molecular oxygen directly from air. The mechanism of BVMOs was shown to proceed analogous to the chemical Baeyer-Villiger oxidation by Ryerson et al. (1982). In type I BVMOs, which represent the majority of known BVMOs, molecular oxygen reacts with reduced FAD in the active site of the enzyme. This leads to the formation of a deprotonated flavin-C4α-peroxide intermediate, which attacks the carbonyl carbon of the substrate upon binding in the active site (Sheng et al. 2001). The thus formed Criegee-intermediate, which exhibits a tetrahedral configuration, rearranges so that the product and water are released. Reduced FAD is regenerated by the NADPH cofactor (Scheme 1b).

The enzymatic mechanism has been studied extensively for cyclohexanone monooxygenase from *Rhodococcus* sp. HI-31 ($CHMO_{Rhodo}$) with several crystal structures showing the movement of enzyme domains during the catalytic cycle (Mirza et al. 2009; Yachnin et al. 2014; Yachnin et al. 2012). However, the first crystal structure of a BVMO was elucidated for phenylacetone monooxygenase from *Thermobifida fusca* (PAMO) (Malito et al. 2004) followed by studies on the mechanism of this enzyme (Orru et al. 2011; Torres Pazmino et al. 2008). These studies highlighted the importance of the catalytic arginine residue R337 by providing crystal structures of key steps in the mechanism. Fig. 1 shows the three-dimensional structure of PAMO including FAD- and NADP-binding domains and the cofactors $NADP^+$ and FAD, as well as relevant active site residues.

PAMO is solvent- and thermostable (Beneventi et al. 2013; de Gonzalo et al., 2006a; Fraaije et al. 2005; Secundo et al. 2011), accepts small aromatic ketones and sulfides (de Gonzalo et al. 2005; Rodriguez et al. 2007) and shows low activity with linear ketones (Fraaije et al. 2005). In comparison to the substrate profile of other well known BVMOs, like the CHMO from *Acinetobacter* sp. strain NCIMB 9781 ($CHMO_{Acineto}$) and cyclopentanone monooxygenases from *Comamonas* sp. strain NCIMB 9782 (CPMO), the substrate scope of PAMO is rather narrow (Fink et al. 2012; Riebel et al. 2012). $CHMO_{Acineto}$ is the most extensively studied BVMO and catalyzes a multitude of reactions enantioselectively (Walsh and Chen 1988). However, its low stability is a major disadvantage in the application of this BVMO. Apart from the application of BVMOs for the production of chiral lactones, which can be used as building blocks, valuable sulfoxides, such as the drug esomeprazole, can be produced by BVMOs. Quite a few comprehensive

Abbreviations: BVMO, Baeyer-Villiger monooxygenase; CHMO, cyclohexanone monooxygenase; CPMO, cyclopentanone monooxygenase; DKCMO, diketocamphane monooxygenase; HAPMO, 4-hydroxyacetophenone monooxygenase; OTEMO, 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase; PAMO, phenylacetone monooxygenase; YMOA, *Yarrowia* monooxygenase A

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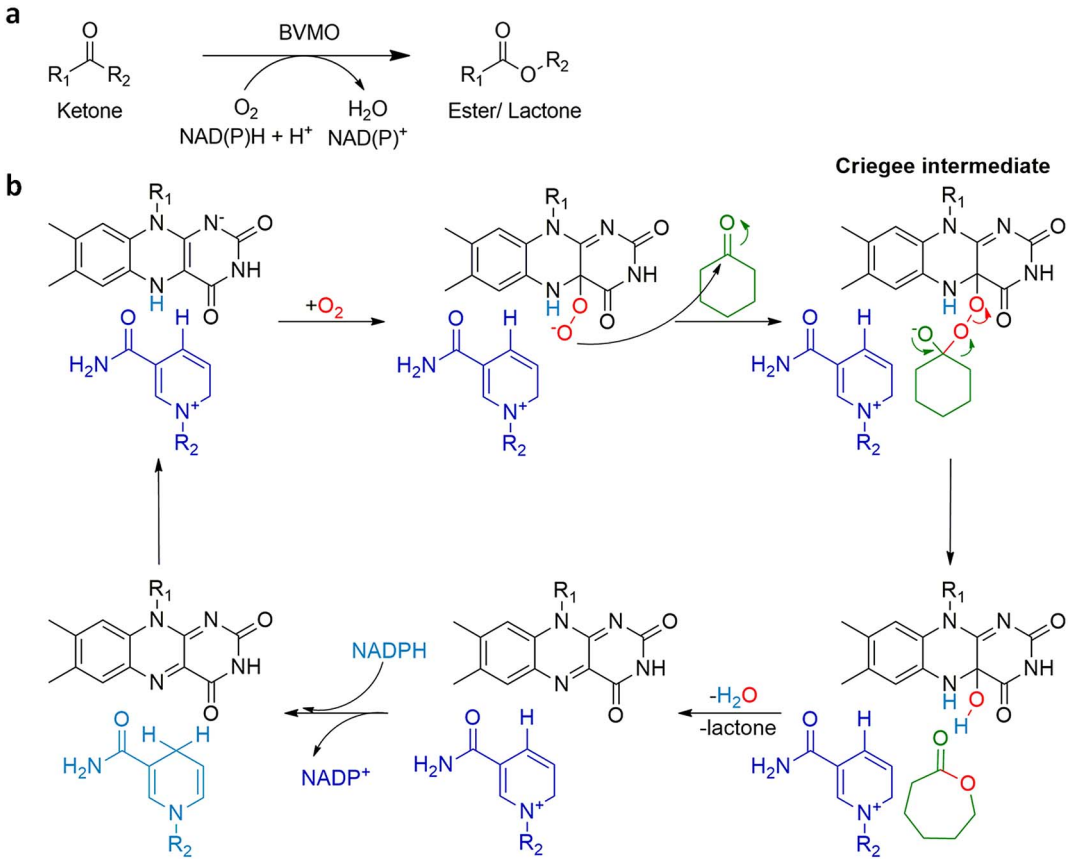

Scheme 1. a) The classical reaction catalyzed by Baeyer-Villiger monooxygenases is the conversion of ketones to esters or lactones by the insertion of an oxygen atom supplied from air. b) Enzymatic mechanism of the BVMO reaction.

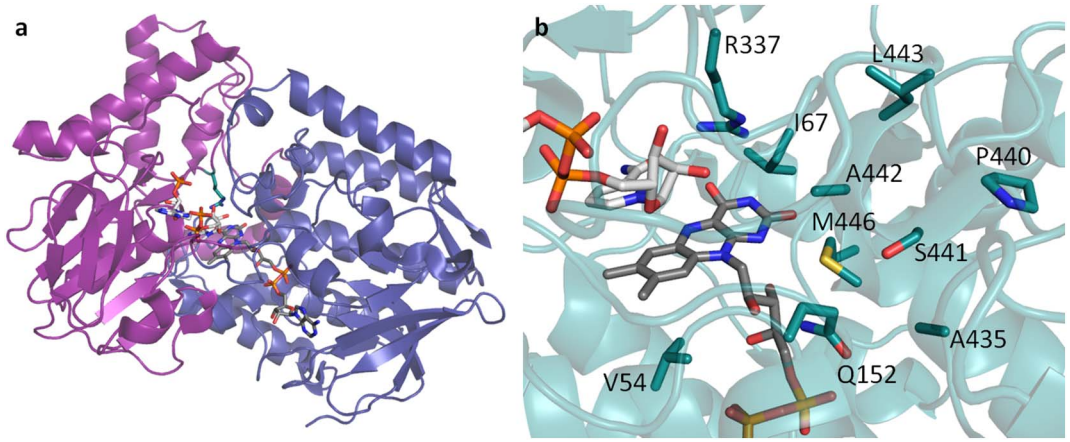

Fig. 1. Structure of phenylacetone monooxygenases (PAMO). a) Three-dimensional structure of PAMO showing FAD (dark grey), $NADP^+$ (light grey) and R337 (turquoise) in the active site of the enzyme. FAD-binding domain (10–158 and 390–542) is shown in blue and NADP-binding domain (159–389) is depicted in purple. b) Active site of PAMO showing the cofactors $NADP^+$ (light grey) and FAD (dark grey) and important active site residues (turquoise). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reviews on the application of BVMOs in biocatalysis have been published in the past 7 years (Balke et al. 2012; Bucko et al. 2016; de Gonzalo et al. 2010; Leisch et al. 2011), most of these focusing on accessible reactions and overcoming limitations by the utilization of immobilization methods and process optimization. The possibility of enabling application of BVMOs by means of protein engineering methods has been reviewed in regard to stereo-/site-selectivity (Wang et al. 2017; Zhang et al. 2012). Apart from improving the enantioselectivity and the substrate scope of BVMOs, the regioselectivity, cofactor usage and heteroatom oxidation have also been targeted.

There are different approaches when performing protein engineering studies depending on the available knowledge of the enzyme's structure and mechanism (Fig. 2) (Bornscheuer and Kazlauskas 2011; Buchholz et al. 2012). If little is known about the enzyme of interest, random mutagenesis of the gene of interest by methods like error-prone PCR (epPCR) is the only choice for protein engineering. This necessitates a massive subsequent screening effort in order to identify the best mutant. The most frequently used high-throughput screening methods are spectrophotometric and fluorimetric assays performed in microtiter plates as they allow an efficient evaluation of a high number of variants.