Structural and Catalytic Characterization of a Fungal Baeyer-Villiger Monooxygenase

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Abstract

Baeyer-Villiger monooxygenases (BVMOs) are biocatalysts that convert ketones to esters. Due to their high regio-, stereo- and enantioselectivity and ability to catalyse these reactions under mild conditions, they have gained interest as alternatives to chemical Baeyer-Villiger catalysts. Despite their widespread occurrence within the fungal kingdom, most of the currently characterized BVMOs are from bacterial origin. Here we report the catalytic and structural characterization of BVMOAFL838 from *Aspergillus flavus*. BVMOAFL838 converts linear and aryl ketones with high regioselectivity. Steady-state kinetics revealed BVMOAFL838 to show significant substrate inhibition with phenylacetone, which was more pronounced at low pH, enzyme and buffer concentrations. Para substitutions on the phenyl group significantly improved substrate affinity and increased turnover frequencies. Steady-state kinetics revealed BVMOAFL838 to preferentially oxidize aliphatic ketones and aryl ketones when the phenyl group are separated by at least two carbons from the carbonyl group. The X-ray crystal structure, the first of a fungal BVMO, was determined at 1.9 Å and revealed the typical overall fold seen in type I bacterial BVMOs. The active site Arg and Asp are conserved, with the Arg found in the “in” position. Similar to phenylacetone monooxygenase (PAMO), a two residue insert relative to cyclohexanone monooxygenase (CHMO) forms a bulge within the active site. Approximately half of the “variable” loop is folded into a short α-helix and covers part of the active site entry channel in the non-NADPH bound structure. This study adds to the current efforts to rationalize the substrate scope of BVMOs through comparative catalytic and structural investigation of different BVMOs.

Introduction

Baeyer-Villiger monooxygenases (BVMOs) are flavin-dependent enzymes that catalyze the conversion of ketones to esters using NAD(P)H and molecular oxygen [1–4]. In addition to this typical reaction, they can also catalyze heteroatom oxidation, including sulfoxidation and N-oxidation, as well as epoxidation reactions. The substrate scope of the collective BVMO family of enzymes has grown to include a variety of substrates ranging from acetone to larger ketones such as steroids. The mild reaction conditions and often high regio-, stereo- and enantioselectivity have made them very attractive as an alternative to chemical Baeyer-Villiger catalysts. Indeed, many directed evolution studies have been performed to increase or alter the substrate scope as well as improve the selectivity and specificity of these enzymes [5,6].
Although the available cloned BVMOs have grown significantly over the past few years, it is only recently that BVMOs from fungal sources have been explored [7,8] despite their widespread presence in the fungal kingdom as revealed through whole-genome sequencing [9]. To date however, the three-dimensional crystal structures of only four distinct bacterial type I Baeyer-Villiger monooxygenases have been determined: phenylacetone monooxygenase (PAMO) from Thermobifida fusca [10], cyclohexanone monooxygenase (CHMO) from Rhodococcus sp. strain HI-31 [11], steroid monooxygenase (STMO) from Rhodococcus rhodochrous [12] and 2-oxo-Δ3,4,5,5-trimethylcyclopentenylacetyl-coenzyme A monooxygenase (OTEMO) from Pseudomonas putida ATCC 17453 [13]. Through extensive structural investigations of these enzymes with bound co-factors, inhibitors, substrates and products, the reaction mechanism of BVMOs has been explained [14–16]. Catalysis of BVMOs involves extensive backbone conformational changes and cofactor movement. In short, NADPH is bound to the BVMO in the “open” conformation, where after the non-covalently bound FAD is reduced and subsequently reacts with molecular oxygen to form the reactive peroxyflavin species. Following substrate entry, the BVMO undergoes a domain rotation and movement of the NADP⁺ to stabilize the peroxyflavin. This is accompanied/mediated by the structuring of a disorderd surface loop. The BVMO, now in a “closed/tight” conformation, reorganizes to the “rotated” conformation through the rotation of the NADP⁺ to allow the substrate to move into the catalytic position. Nucleophilic attack with formation of the Criegee intermediate occurs in this “rotated” conformation. Following the production of the lactone product, the BVMO returns to a “closed/tight”-like NADP⁺ conformation followed by release of the product in the “loose” conformation.

Despite these informative studies, the basis of substrate acceptance and specificity, especially of larger substrates, is still not completely understood. PAMO has a rather limited substrate scope of mostly phenyl substituted linear ketones [17] while STMO can only convert both progesterone and phenylacetone [12,18]. In contrast CHMO has an extremely wide substrate scope [3]. We have recently reported on four closely related BVMOs from Aspergillus flavus with distinct substrate profiles [7]. Amongst the four BVMOs described, BVMOAFL838 showed the best conversion of alkanones with chain lengths of C8-C12, but was unable to convert most of the cyclic ketones tested. Here we describe the catalytic and structural characterization of BVMOAFL838. This structure represents the first fungal BVMO solved and contributes to the efforts to rationalize the substrate specificity of BVMOs.

Materials and Methods

Strains and Vectors

BVMOAFL838 was heterologously expressed from the pET-22b(+) vector (Novagen) in E. coli BL21Gold(DE3) (Stratagene). The previously constructed plasmid [7] served as a template to construct a C-terminally histidine (CTH) tagged variant of BVMOAFL838 by deleting the stop codon and plasmid backbone between the gene and the plasmid’s six histidine’s codons. This variant was prepared as previously described [19] using the primers BVMO_Histag_F (5’ CAC CAC CAC CAC CAC TGA GAT C 3’) and BVMO_Histag_R (5’ TGC TTT CGC AAA ACC AAA GAA ATC CTC 3’). Bacillus megaterium glucose dehydrogenase (BmGDH) was kindly provided by Dr. Dirk Holtmann (Dechema, Germany) in the pETDuet vector (Novagen) cloned in the second multiple cloning site via NdeI and XhoI. The BmGDH ORF was subsequently sub-cloned to pET-28b(+) to generate an N-terminally histidine tagged variant using the same restriction sites. The BVMOAFL838_K511A mutant was prepared using site-directed mutagenesis with the QuickChange (Stratagene) method using primers K511A_F (5’ CAA CAT TCC GGG CGC GCC TGT CTC ATC ATC 3’) and a complementary reverse primer.
E. coli strains were routinely grown in LB medium containing ampicillin (0.1 mg.mL\(^{-1}\)) or kanamycin (0.03 mg.mL\(^{-1}\)) at 37°C with shaking (200 rpm).

**Protein Expression and Purification**

E. coli BL21Gold(DE3) harbouring the pET-22:BVMO\(_{AFL388CTH}\) or pET-28:BmGDH plasmid was inoculated into ZYP5052 expression medium \[20\] containing 0.1 mg.mL\(^{-1}\) ampicillin or 0.03 mg.mL\(^{-1}\) kanamycin respectively. Cells were grown for 24–48 h at 20°C where after they were harvested through centrifugation (8 000 xg, 10 min) and resuspended in 50 mM Tris-HCl (pH 7.4) buffer containing 0.5 M NaCl and 20–30 mM imidazole (binding buffer). Cells were broken by a single passage through a continuous cell disrupter (Constant Systems) using 30 kPsi at 4°C and the crude cell-free extract was obtained through ultracentrifugation (100 000 xg, 90 min). The soluble fraction was loaded onto a 5 mL HisTrap FF Ni-affinity column (GE Healthcare) equilibrated in binding buffer. Unbound proteins were removed by washing with 10 column volumes of the same buffer. Proteins were eluted in the same buffer using a linear gradient of increasing imidazole concentration. Fractions containing the protein of interest were pooled and concentrated to approximately 2 mL through ultrafiltration using a 30 kDa NMWL Amicon Ultrafiltration unit. The concentrated BVMO\(_{AFL388CTH}\) protein was soaked overnight in excess FAD. Concentrated protein samples were loaded onto a Sephacryl S100HR size exclusion column (GE Healthcare). Proteins were eluted in 10 mM Tris-HCl buffer (pH 8) containing 100 mM NaCl. Purified proteins were evaluated on SDS-PAGE using PageRuler Prestained Protein Ladder (ThermoScientific) and stained with Coomassie brilliant blue R-250. Protein concentrations were determined using the Bradford protein assay (Bio-Rad). FAD content was determined by incubation of the protein sample (typically 5 mg.mL\(^{-1}\)) in ~ 8 M urea for 30 min, followed by spectrophotometric quantification using the extinction coefficient of 11.3 mM\(^{-1}\).cm\(^{-1}\) at 450 nm.

**Biotransformations and Steady-state kinetics**

Biotransformations were performed in amber glass vials (40 mL) in a total reaction volume of 1 mL. Whole-cell (WC) and cell-free extract (CFE) biotransformations were performed as previously described \[7\]. Reactions with purified BVMO were performed in 100 mM Tris-HCl buffer (pH 8) containing 2 μM BVMO, 0.5 U BmGDH, 100 mM glucose, 0.3 mM NADP\(^{+}\) and 10 mM substrate. Reactions were maintained at 20°C with shaking (200 rpm), where after they were extracted using an equal volume (2 x 0.5 mL) ethyl acetate containing either 2 mM 1-undecanol or 3-octanol as internal standard. GC-FID (and GC-MS for product identification) was performed on a Finnigan Trace GC ultra (ThermoScientific) equipped with a FactorFour VF-5ms column (60 m x 0.32 mm x 0.25 μm, Varian). Steady-state kinetics were performed by monitoring the oxidation of NADPH spectrophotometrically at 340 nm (ε\(_{340}\) = 6.22 mM\(^{-1}\).cm\(^{-1}\)) or 370 nm (ε\(_{370}\) = 2.70 mM\(^{-1}\).cm\(^{-1}\)). To investigate optimal pH, temperature, stability and effect of organic solvents, reactions typically contained 2 μM BVMO, 0.3 mM NADPH, 1 mM phenylacetone, 1% (v/v) methanol (100 mM Tris-HCl, pH 8; 25°C).

**Crystallization and Structure Determination**

Crystals were grown using hanging-drop vapour-diffusion in 1 μL drops consisting of equal volumes of 6 mg.mL\(^{-1}\) BVMO and reservoir solution (0.1 M Tris-HCl pH 9, 1.8 M ammonium sulphate) and 5 x molar excess of FAD and NADP\(^{+}\) at 289 K. Yellow crystals grew within 2 weeks. Crystals were soaked in reservoir solution containing 30% glycerol prior to cryocooling. X-ray diffraction data (Table 1) were collected at Diamond (UK) on beamline I04-1. Data was...
processed using MOSFLM [21] and POINTLESS [22], with intensities scaled and merged using SCALA [22] from the CCP4 suite of programs. Molecular replacement was performed using Phaser [23] with PAMO (PDB:1W4X) as search model. Refinement was performed through iterative cycles of manual model building in COOT [24] and TLS and restrained refinement using Refmac [25]. Structures were validated using programs within the CCP4 suite [26]. Figures were generated in PyMOL.

Accession numbers
Coordinates and structure factors for \( \text{BVMO}_{\text{AFL838}} \) have been deposited in the Protein Data Bank (PDB) under accession number 5J7X.

Results and Discussion
Purification and Characterization
\( \text{BVMO}_{\text{AFL838}} \) was heterologously expressed in \textit{E. coli} as a C-terminally 6x histidine tagged protein and purified to near homogeneity using Ni-affinity and size exclusion chromatography (SEC). SDS-PAGE analysis confirmed the monomer molecular weight of 62.2 kDa (Fig 1A). SEC also showed \( \text{BVMO}_{\text{AFL838}} \) to exist as a monomer in solution. \( \text{BVMO}_{\text{AFL838}} \) was recovered with only 70–80% of bound flavin, but near full occupancy of the FAD could be achieved by soaking with excess FAD before SEC (Fig 1B). \( \text{BVMO}_{\text{AFL838}} \) was optimally active at pH 9, but retained approximately 80% activity at pH 8.8–8.5 and 60% at pH 7.5 and 9.5. Although the optimal temperature for \( \text{BVMO}_{\text{AFL838}} \) was found to be 40°C, the enzyme was rapidly inactivated at 40°C, with a half-life of only 21 min (Fig 2). Of the organic solvents tested, methanol showed to least affect activity, with nearly a 100% relative activity in up to 5% (v/v).

Table 1. Data Collection and Refinement Statistics.

| Data Collection and Processing |          |
|-------------------------------|----------|
| Beamline                      | I04-I    |
| Wavelength (Å)                | 0.9174   |
| Resolution (Å)                | 32.4–1.9 (2.0–1.9) |
| Space group                   | C 2 2 2  |
| Unit cell parameters          | a = 109.67Å b = 177.51Å c = 76.37Å α = 90° β = 90° γ = 90° |
| Unique reflections            | 58733 (8465) |
| Completeness (%)              | 99.6 (99.6) |
| Mn(I)/sd(I)                   | 15.3 (3.0) |
| Multiplicity                  | 6.2 (6.3) |
| \( R_{merge} \)               | 0.081 (0.669) |

| Refinement                    |          |
|-------------------------------|----------|
| \( R_{w} einmal \)/\( R_{free} \) a | 0.1571/0.2046 |
| Molecules in ASU               | 1        |
| Average B-factor, all atoms (Å²) | 28.0 |
| r.m.s.d. Bond lengths (Å)      | 0.031    |
| r.m.s.d. Bond angles (°)       | 2.71     |
| Ramachandran distribution (%)  | 96.9/2.7/0.4 |

Values for the highest resolution shell given in parenthesis
a \( R_{free} \) calculated from 5% reflections omitted from structure refinement
b Favoured/Permitted/Disallowed
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We have previously shown BVMO AFL838 to be active towards aliphatic ketones and aryl ketones, with very few monocyclic ketones converted during whole-cell biotransformations [7]. Purified enzyme was tested using glucose dehydrogenase from *Bacillus megaterium* (*Bm*GDH) for cofactor regeneration to eliminate the possible uptake/transport limitations or toxicity of substrates or products toward *E. coli* whole cells. Similar to the whole-cell biotransformations which we previously reported, efficient conversion of the 2-alkanones was observed with nearly complete conversion of the C8-C12 substrates (10 mM of 1a, 3-5a) within 8 h and even within 2 h for C8 (1a), with absolute regioselectivity forming the alkyl acetate products (Fig 3, Table 2). Turnover frequency (TOF) decreased with increasing chain-length but this could possibly be attributed to a decrease in solubility. Complete conversion of 3-octanone (2a) was also observed after 2 h, and observed TOF ($k_{obs}$) values for 10 mM 2-octanone (1a) and 3-octanone were 5.3 s⁻¹ and 6.6 s⁻¹ respectively. To better investigate the effect of the position of the ketone group, steady-state kinetics were performed (Fig 4). A much lower $K_M$ was observed for 2-octanone compared to 3-octanone, and although both substrates suffered from substrate inhibition, 2-octanone’s inhibition was more pronounced ($K_i = 1.5 ± 0.4$ mM) leading to lower TOF at high (10 mM) substrate concentrations (Table 2). Conversions of aliphatic
ketones with substrate concentrations of up to 25 mM have been reported for BVMO3 from *Dietzia* sp. D5 [27], however no kinetic data was reported to evaluate reaction rates or possible substrate inhibition.

Investigation into BVMOAFL838’s affinity for longer chain 2-alkanones (3-5a) revealed $K_m$ values lower than 0.01 mM and less substrate inhibition than with 2-octanone with TOF reaching 6.8 s⁻¹ (6.6 U.mg⁻¹) for 2-decanone at 10 mM. Wild-type PAMO has been shown to accept aliphatic ketones [17] such as 2-dodecanone with a turnover frequency of 0.23 s⁻¹ ($K_m = 0.26$ mM) and a quadruple mutant (15-F5) with improved activity towards 2-octanone ($k_{cat} = 2.3$ s⁻¹, $K_m = 0.25$ mM) has also been reported [28]. BVMOs from *Pseudomonas putida* KT2440 [29] and *P. veronii* MEK700 (MekA) [30] have also been shown to efficiently convert various aliphatic ketones, with specific activities for MekA between 0.45–0.89 U.mg⁻¹ for C8-C12 2-alkanones.

A good correlation was observed between whole-cell biotransformations and biotransformations using purified enzymes for aryl ketones, except no conversion was observed for phenylacetone (8a) and benzaldehyde (12a) using purified enzyme (Fig 5, Table 3). Very low TOF (0.3 s⁻¹) were observed against 10 mM phenylacetone and steady-state kinetics revealed severe substrate inhibition with phenylacetone especially at lower enzyme concentrations (Fig 6A). Incubation of BVMOAFL838 with excess phenylacetone followed by reduction with NADPH did not affect the initial FAD reduction (NADPH binding), suggesting inhibition occurs during one of the intermediate steps of catalysis. Substrate inhibition by some acetophenone and benzaldehyde derivatives have also been observed with HAPMO [31] with a strong correlation between substrate affinity ($K_m$) and degree of substrate inhibition. Substrate inhibition of BVMOAFL838 was also more pronounced at lower buffer concentrations and pH values (Fig 6B and 6C) to an extent that no observable rate could be detected. Glucose dehydrogenase (GDH) was used for cofactor regeneration in the biotransformations with purified enzyme and GDH is known to rapidly acidify the reaction mixture [32]. This type of fast acidification is not found with whole-cell biotransformation using glucose for cofactor regeneration through central metabolism. In addition, higher enzyme concentrations in whole-cells and potentially reduced effective substrate concentration due to diffusion/uptake limitations to the cytoplasm may also alleviate the substrate inhibition observed when using purified enzyme. The initial rates (measured as NADPH oxidation) were not maintained even at high substrate concentrations, suggesting also product inhibition.

| Substrate | Conversion (%) | TOF (s⁻¹) | $K_m$ (mM) |
|-----------|---------------|-----------|------------|
| 2h        | 8h            | 10 mM $k_{obs}$ | |
| 1a 2-octanone | >99 | >99 | 5.3 | 0.01 ± 0.003 |
| 2a 3-octanone | >99 | >99 | 6.6 | 0.17 ± 0.03 |
| 3a 2-decanone | 87 | >99 | 6.8 | <0.01 |
| 4a 2-undecanone | 42 | 98 | 6.1 | <0.01 |
| 5a 2-dodecanone | 21 | 99 | 5.3 | <0.01 |

Table 2. Biotransformations of aliphatic ketones (10 mM) by purified BVMOAFL838 (2 μM) and kinetic parameters determined from initial velocities of NADPH oxidation.
Similarly, no conversion was observed with benzaldehyde (12a) as substrate using purified enzyme although more than 60% conversion was observed using whole-cell biotransformations. Steady-state kinetics gave very low TOFs but no substrate inhibition was observed up to a concentration of 30 mM. Only slight substrate inhibition ($K_i = 41 \pm 6$ mM) was observed with the para-substituted 4-methoxy benzaldehyde (13a) with more than 80% conversion observed after 2 h. Likewise, acetophenone with a hydroxyl substitution on the para position (7a) showed complete conversion after only 2 h, compared to 4% for acetophenone (6a). The substitutions on the phenyl ring lowered the $K_m$ values with more than an order of magnitude, with TOF increased to more than 1 s$^{-1}$ (Table 3). Increasing the distance between the ketone and phenyl group by a single carbon (9-11a), dramatically increased BVOAOFL838’s affinity for the substrates, with TOF now between 6 and 8 s$^{-1}$ at 0.1 mM (2.4–4.3 s$^{-1}$ at 10 mM) and again the para-substituted substrates (10a and 11a) giving higher rates. Similar observations that substituents on the para position appear to be critical for substrate recognition have been made for 4-hydroxyacetophenone monooxygenases (HAPMOs) from both Pseudomonas fluorescens ACB [33,34] and Pseudomonas putida JD1 [35].

Absolute regioselectivity was also observed against all the aryl ketones tested. The ester products (12b, 13b) from benzaldehyde and 4-methoxybenzaldehyde were rapidly hydrolyzed to form phenol and 4-methoxyphenol (mequinol) as sole products. This regioselectivity is again similar as to what has been observed for HAPMO with various benzaldehyde derivatives [31], where even benzaldehydes with electron-withdrawing substituents on the aromatic ring prefers the formation of phenols.

**Overall structure of BVOAOFL838**

The crystal structure of BVOAOFL838, the first of a fungal BVMO, was determined at a resolution of 1.9 Å. BVOAOFL838 shares more than 40% sequence identity with PAMO, which was

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For the full details and further references, please refer to the original publication.
used as search model for molecular replacement. Despite numerous attempts to co-crystallize BVMOAFL838 with co-factor and substrates, none of the crystals analyzed showed any electron density for bound NADP+ or the substrates used. A similar observation was made during the crystallization of PAMO [10], where the excess ammonium sulphate used as precipitant (crystallization agent) prevents the binding of NADP+. The first eight amino acids of the N-terminus were not modelled as it was not resolved in the electron density. Electron density was also not found for residues 230–239 which typically folds into a short α-helix on the surface of other BVMOs on the opposite side of the substrate entry (NADPH binding) channel.

BVMOAFL838, similar to the four bacterial BVMOs, displays two Rossmann fold domains, representing the FAD and NADP binding domains, with the isoalloxazine ring of FAD positioned at the domain interface (Fig 7A). The FAD is bound with the re-face exposed to the substrate binding pocket and the si-face on top of a conserved Tyr69 and Trp52 forming conserved hydrogen bond interactions with the ribitol moiety of FAD. Except for the conserved Rossmann fold signature motif (GXGXXG) the region binding the rest of the FAD molecule is less conserved as most of the contacts are with main chain amide groups and via water mediated interactions. Despite this, Val115 is absolutely conserved between the five known BVMO

Table 3. Biotransformations of aryl ketones and aldehydes by E. coli whole cells expressing BVMOAFL838 (WC) and purified BVMOAFL838 (ENZ).

| Substrate                  | Conversion (%) | TOF (s⁻¹) | K_m (mM) |
|----------------------------|----------------|-----------|----------|
|                            | WC 2h | ENZ 2h | ENZ 8h | 10 mM k_{obs} |          |
| 6a Acetophenone            | 19    | 4      | 13     | 0.05           | 10.0 ± 1.2 |
| 7a 4-Hydroxyacetophenone   | >99   | >99    | >99    | 1.4            | 0.8 ± 0.05  |
| 8a Phenylacetone           | 72    | n.c.   | n.c.   | 0.28²         | 2.4 ± 1.2² |
| 9a 4-Phenyl-2-butanone     | 64    | >99    | >99    | 2.4            | <0.01³     |
| 10a 4-(4-Hydroxyphenyl)-2-butanone | 85 | >99 | >99 | 2.8 | <0.01³ |
| 11a 4-(4-Methoxyphenyl)-2-butanone | 68 | 85 | 85 | 4.3 | <0.01³ |
| 12a Benzaldehyde           | 62    | n.c.   | n.c.   | 0.03           | 6.5 ± 0.7  |
| 13a 4-Methoxybenzaldehyde  | 63    | 81     | 88     | 1.0            | 0.03 ± 0.002 |

² Determined using 2 μM of BVMO
³ Substrate inhibition observed, K_i >> K_m

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Fig 6. Steady-state kinetics of BVMOAFL838 with phenylacetone at different enzyme concentrations (A), Tris buffer concentrations (B) and pH values (C).
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structures, with two hydrogen bonds forming between the adenine of FAD and the main chain amide and carbonyl group of Val. The previously identified BVMO-signature motif [36] is absolutely conserved in position and sequence.

Active site architecture

The catalytic site Arg337 and Asp63, implicated in stabilization of the peroxyflavin intermediate and positioning and guiding the cofactor and substrate during catalysis, are conserved. The Arg is located in the “in” position and forms two salt bridges with Asp (Fig 7B). Similar to PAMO, a bulge (residues 442–444) protrudes into the active site of BVMO AFL838 (Fig 7B). Mutation studies showed that elimination of this bulge increased the substrate scope of PAMO [37]. However, STMO and OTEMO display similar bulges, but accept progesterone and small monocyclic ketones as substrates respectively.

Variable loop structure

Partially covering the substrate entry channel of BVMO AFL838 is a loop region (residues 449 to 514) of which the first half forms an α-helix structure (Fig 7C). This mobile loop region is
typically disordered (CHMO, STMO) or forms an unstructured loop (PAMO) or a β-hairpin structure (OTEMO) that is solvent exposed and positioned away from the active site channel in the “resting” state. In BVMO_{AFL838}, the mobile/“variable” loop adopts a position similar to that in the CHMO “closed” structure, where part of the active site entry channel is closed off. In this conformation however, the conserved Trp502 (W492 CHMO numbering) is positioned away from the active site and the NADPH cofactor, suggesting a rearrangement of this loop upon cofactor and substrate binding. Apart from Arg214 and Lys336 that have been implicated in NADPH binding, the conformation of the mobile loop also positioned Lys511 within close proximity to the anticipated 2’-phosphate of NADPH as suggested through superimposition with other BVMOs. To probe whether the conformation of the variable/mobile loop observed in the structure is physiologically important, K511 was mutated to an Ala. Although still catalytically active, the K511A mutant showed reduced catalytic activity towards 3-octanone as well as an increased uncoupling of NADPH oxidation and product formation.

Conclusions

BVMO_{AFL838} has a clear preference towards aliphatic ketones and phenyl substituted 2-alkanones (aryl ketones where the alkyl chain is at least two carbons between the phenyl and carbonyl group). To our knowledge, this data represents the highest specific rates obtained to date for BVMO transformations of 2-alkanones. Moreover, biotransformations of linear and aryl ketones using purified enzyme compared to whole-cell biotransformations highlights the importance of kinetic studies as opposed to single-concentration conversions to determine the substrate scope and preference of BVMOs when working with purified enzymes as these systems are more prone to substrate inhibition. BVMO_{AFL838} constitutes the first structural investigation of a fungal BVMO. The crystal structure of BVMO_{AFL838} revealed an overall fold similar to bacterial BVMOs. More extensive crystallization trials is currently underway to find conditions to allow solving BVMO_{AFL838} with NADP⁺ and substrates to further investigate conformations of the “variable” loop region, as well as determinants of substrate scope and regioselectivity. More data is currently needed to understand the discrepancy between substrate pocket plasticity to accommodate a wide range of substrates yet high product selectivity.

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Author Contributions

Conceived and designed the experiments: DJO MSS. Performed the experiments: FMF CT. Analyzed the data: DJO MSS CT FMF. Wrote the paper: DJO MSS CT FMF.

References

1. Kamerbeek NM, Janssen DB, van Berkel WJH, Fraaije MW. Baeyer–Villiger Monooxygenases, an Emerging Family of Flavin-Dependent Biocatalysts. Adv Synth Catal. 2003; 345: 667–678. doi: 10.1002/adsc.200303014
2. Baike K, Kadow M, Mailin H, Saß S, Bornscheuer UT. Discovery, application and protein engineering of Baeyer-Villiger monooxygenases for organic synthesis. Org Biomol Chem. 2012; 10: 6249–6265. doi: 10.1039/c2ob25704a PMID: 22733152
3. Leisch H, Morley K, Lau PCK. Baeyer-Villiger monooxygenases: more than just green chemistry. Chem Rev. 2011; 111: 4165–4222. doi: 10.1021/cr1003437 PMID: 21542563
4. Torres Pazmiño DE, Dudek HM, Fraaije MW. Baeyer-Villiger monoxygenases: recent advances and future challenges. Curr Opin Chem Biol. 2010; 14: 138–144. doi: 10.1016/j.cobp.2009.11.017 PMID: 20015679

5. Zhang Z-G, Parra LP, Reetz MT. Protein engineering of stereoselective Baeyer-Villiger monoxygenases. Chemistry. 2012; 18: 10160–10172. doi: 10.1002/chem.201202163 PMID: 22807240

6. Clouthier CM, Kayser MM, Reetz MT. Designing new Baeyer-Villiger monoxygenases using restricted CASTing. J Org Chem. 2006; 71: 8431–8437. doi: 10.1021/jo0613636 PMID: 17064016

7. Ferroni FM, Smit MS, Opperman DJ. Functional divergence between closely related Baeyer-Villiger monoxygenases from *Aspergillus flavus*. J Mol Catal B Enzym. 2014; 107: 47–54. doi: 10.1016/j.molcatb.2014.05.015

8. Mascotti ML, Juri Ayub M, Dudek H, Sanz MK, Fraaije MW. Cloning, overexpression and biocatalytic exploration of a novel Baeyer-Villiger monoxygenase from *Aspergillus fumigatus* AT293. AMB Express. 2013; 3: 33. doi: 10.1186/2191-0855-3-33 PMID: 23767684

9. Mascotti ML, Lapadula WJ, Juri Ayub M. The origin and evolution of Baeyer—Villiger monoxygenases (BVMOs): An ancestral family of flavin monoxygenases. PLoS One. 2015; 10: e0132689. doi: 10.1371/journal.pone.0132689

10. Malito E, Alfieri A, Fraaije MW, Mattevi A. Crystal structure of a Baeyer-Villiger monoxygenase. Proc Natl Acad Sci U S A. 2004; 101: 13157–13162. doi: 10.1073/pnas.0405381101 PMID: 15328411

11. Mirza IA, Yachnin BJ, Wang S, Grosse S, Bergeron H, Imura A, et al. Crystal structures of cyclohexa-none monoxygenase reveal complex domain movements and a sliding cofactor. J Am Chem Soc. 2009; 131: 8848–8854. doi: 10.1021/ja9010578 PMID: 19385644

12. Francescini S, van Beek HL, Pennetta A, Martinoli C, Fraaije MW, Mattevi A. Exploring the structural basis of substrate preferences in Baeyer-Villiger monoxygenases: insight from steroid monoxygenase. J Biol Chem. 2012; 287: 22626–22634. doi: 10.1074/jbc.M112.372177 PMID: 22605340

13. Leisch H, Shi R, Grosse S, Morley K, Bergeron H, Cygler M, et al. Cloning, Baeyer-Villiger biooxidations, and structures of the camphor pathway 2-oxo-Δ(3)-4,5,5-trimethylcyclopentenylacetoyl-coenzyme A monoxygenase of *Pseudomonas putida* AT17453. Appl Environ Microbiol. 2012; 78: 2200–2212. doi: 10.1128/AEM.07694-11 PMID: 22267661

14. Orru R, Dudek HM, Martinioli C, Torres Pazmiño DE, Royant A, Weik M, et al. Snapshots of enzymatic Baeyer-Villiger catalysis: oxygen activation and intermediate stabilization. J Biol Chem. 2011; 286: 29284–29291. doi: 10.1074/jbc.M111.255075 PMID: 21697090

15. Yachnin BJ, Sprules T, McEvoy MB, Lau PCK, Berghuis AM. The substrate-bound crystal structure of a Baeyer-Villiger monoxygenase exhibits a Criegee-like conformation. J Am Chem Soc. 2012; 134: 7788–7798. doi: 10.1021/ja211876p PMID: 22506764

16. Yachnin BJ, McEvoy MB, MacCuish RJD, Morley KL, Lau PCK, Berghuis AM. Lactone-bound structures of cyclohexanone monoxygenase provide insight into the stereochemistry of catalysis. ACS Chem Biol. 2014; 9: 2843–2851. doi: 10.1021/cb500442a PMID: 25265531

17. Fraaije MW, Wu J, Heuts DPHM, van Hellemont EW, Sterpetti JHL, Janssen DB. Discovery of a thermostable Baeyer-Villiger monoxygenase by genome mining. Appl Microbiol Biotechnol. 2005; 66: 393–400. doi: 10.1007/s00253-004-1749-5 PMID: 15599520

18. Miyamoto M, Matsumoto J, Iwaya T, Itagaki E. Bacterial steroid monoxygenase catalyzing the Baey-er-Villiger oxidation of C21-ketosteroids from *Rhodococcus rhodochrous*: the isolation and characterization. Biochim Biophys Acta. 1995; 1241: 115–124. Available: http://www.ncbi.nlm.nih.gov/pubmed/7669800 PMID: 7669800

19. Opperman DJ, Reetz MT. Towards practical Baeyer-Villiger-monoxygenases: design of cyclohexa-none monoxygenase mutants with enhanced oxidative stability. ChemBiochem. 2010; 11: 2589–2596. doi: 10.1002/cbic.201000464 PMID: 21080396

20. Studier FW. Protein production by auto-induction in high-density shaking cultures. Protein Expr Purif. 2005; 41: 207–234. doi: 10.1016/j.pep.2005.01.016 PMID: 15915565

21. Leslie AGW, Powell HR. Processing diffraction data with MOSFLM. Evolving methods for macromolecular crystallography. 2007; 245: 45–51. doi: 10.1007/978-1-4020-6316-9_4

22. Evans P. Scaling and assessment of data quality. Acta Crystallogr Sect D Biol Crystallogr. International Union of Crystallography; 2006; 62: 72–82. doi: 10.1107/S0907444905036693

23. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. J Appl Crystallogr. International Union of Crystallography; 2007; 40: 658–674. doi: 10.1107/S0021889807021206 PMID: 19461840

24. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. Acta Crystallogr Sect D Biol Crystallogr. International Union of Crystallography; 2010; 66: 486–501. doi: 10.1107/S0907444910007493
25. Murshudov GN, Skubák P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, et al. REFMAC 5 for the refinement of macromolecular crystal structures. Acta Crystallogr Sect D Biol Crystallogr. 2011; 67: 355–367. doi:10.1107/S0907444911001314

26. Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, et al. Overview of the CCP 4 suite and current developments. Acta Crystallogr Sect D Biol Crystallogr. International Union of Crystallography; 2011; 67: 235–242. doi:10.1107/S0907444910045749

27. Bisagni S, Smuś J, Chávez G, Hatti-Kaul R, Mamo G. Cloning and expression of a Baeyer–Villiger monooxygenase oxidizing linear aliphatic ketones from Dietzia sp. D5. J Mol Catal B Enzym. 2014; 109: 161–169. doi:10.1016/j.molcatb.2014.08.020

28. Dudek HM, Fink MJ, Shivanje AV, Dennig A, Mihovilovic MD, Schwaneberg U, et al. Extending the substrate scope of a Baeyer-Villiger monooxygenase by multiple-site mutagenesis. Appl Microbiol Biotechnol. 2014; 98: 4009–4020. doi: 10.1007/s00253-013-5364-1 PMID: 24247989

29. Rehdorf J, Kirschner A, Bornscheuer UT. Cloning, expression and characterization of a Baeyer-Villiger monooxygenase from Pseudomonas putida KT2440. Biotechnol Lett. 2007; 29: 1393–1398. doi: 10.1007/s10529-007-9401-y PMID: 17530181

30. Völker A, Kirschner A, Bornscheuer UT, Altenbuchner J. Functional expression, purification, and characterization of the recombinant Baeyer-Villiger monooxygenase MekA from Pseudomonas veronii MEK700. Appl Microbiol Biotechnol. 2008; 77: 1251–1260. doi: 10.1007/s00253-007-1264-6 PMID: 18034235

31. Moonen MJH, Westphal AH, Rietjens IMCM, Van Berkel WJH. Enzymatic Baeyer-Villiger oxidation of benzaldehydes. Adv Synth Catal. 2005; 347: 1027–1034. doi:10.1002/adsc.200404307

32. Pham SQ, Gao P, Li Z. Engineering of recombinant E. coli cells co-expressing P450pyrTM monooxygenase and glucose dehydrogenase for highly regio- and stereoselective hydroxylation of alicyclics with cofactor recycling. Biotechnol Bioeng. 2013; 110: 363–373. doi:10.1002/bit.24632 PMID: 22886996

33. Kamerbeek NM, Moonen MJ, Van Der Ven JG, Van Berkel WJ, Fraaije MW, Janssen DB. 4-Hydroxyacetophenone monooxygenase from Pseudomonas fluorescens AOB. A novel flavoprotein catalyzing Baeyer-Villiger oxidation of aromatic compounds. Eur J Biochem. 2001; 268: 2547–2557. doi:10.1046/j.1432-1327.2001.02137.x PMID: 11322873

34. Kamerbeek NM, Olsthoorn AJJ, Fraaije MW, Janssen DB. Substrate specificity and enantioselectivity of 4-hydroxyacetophenone monooxygenase. Appl Environ Microbiol. 2003; 69: 419–426. doi:10.1128/AEM.69.1.419 PMID: 12514023

35. Rehdorf J, Zimmer CL, Bornscheuer UT. Cloning, expression, characterization, and biocatalytic investigation of the 4-hydroxyacetophenone monooxygenase from Pseudomonas putida JD1. Appl Environ Microbiol. 2009; 75: 3106–3114. doi: 10.1128/AEM.02707-08 PMID: 19251899

36. Fraaije MW, Kamerbeek NM, van Berkel WJH, Janssen DB. Identification of a Baeyer-Villiger monooxygenase sequence motif. FEBS Lett. 2002; 518: 43–47. doi:10.1016/S0014-5793(02)02623-6 PMID: 11997015

37. Boccola M, Schulz F, Leca F, Vogel A, Fraaije MW, Reetz MT. Converting Phenylacetone Monooxygenase into Phenylcyclohexanone Monooxygenase by Rational Design: Towards Practical Baeyer-Villiger Monooxygenases. Adv Synth Catal. 2005; 347: 979–986. doi:10.1002/adsc.200505069