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Research Paper

Fungal BVMOs as alternatives to cyclohexanone monooxygenase

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ABSTRACT

FAD-dependent Baeyer-Villiger monooxygenases (BVMOs) have proven to be useful biocatalysts in the selective and specific oxygenation of various ketones. Despite the cloning, heterologous expression and characterization of close to 80 members of this enzyme family, some sub-groups of BVMOs still remain underrepresented and their evolutionary relationship uncertain. Until recently, very few fungal BVMOs have been described. Our previous investigations into BVMOs from the fungus *Aspergillus flavus*, yielded very little activity on simple cyclic ketones. Here we report on another four BVMOs from *A. flavus* that are more closely related to cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871. Evolutionary analysis with other characterized BVMOs show their closest relationship to be with either cycloalkanone monooxygenase (CAMO) or 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-coenzyme A monooxygenase (OTEMO). The OTEMO-related $BVMO_{AFL706}$ and $BVMO_{AFL334}$ were heterologously expressed in *E. coli*, purified and shown to be able to convert a range of cyclic and substituted cyclic ketones. Of the unsubstituted cyclic ketones, cyclohexanone showed the highest conversion with maximum turnover frequencies reaching 4.3 s^{-1} for $BVMO_{AFL706}$. Unlike $CHMO_{acinet}$, and many of the closely related BVMOs, no substrate inhibition was observed with cyclohexanone to a concentration of up to 30 mM, creating the possibility for applications requiring high substrate loading. Aliphatic ketones were also readily converted with excellent regioselectivity. Similar to $CHMO_{acinet}$, acetophenones were not converted and the oxidation of *rac-cis*-bicyclo[3.2.0]hept-2-en-6-one occurs enantiodivergently, with the (1*R*,5*S*) isomer converted to the "normal" lactone and the (1*S*,5*R*) isomer to the "abnormal" lactone.

1. Introduction

Type I Baeyer-Villiger monooxygenases (BVMOs) are FAD-dependent enzymes that catalyze the conversion of cyclic and linear ketones to lactones and esters, respectively, utilizing NADPH as electron donor and molecular oxygen as oxidant [1–4]. Furthermore, they can catalyze heteroatom oxidation, including sulfoxidations and N-atom hydroxylations [5–8], as well as epoxidations of C—C double bonds [9] and either ester or fatty acid formation from aldehydes [10,11]. The catalytic potential of BVMOs have been demonstrated not only as single-step regio- and enantiospecific biotransformations of various substrates, but also as part of enzymatic cascade/tandem reactions [12–16] and chemo-enzymatic strategies [17,18], especially for the production of ε-caprolactone [19–26].

Cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871 ($CHMO_{acinet}$) [27–29] still remains one of the most widely studied BVMOs and has been subjected to various directed evolution studies to further improve or change its activity and/or selectivity [30–32]. The catalytic versatility and high selectivity and specificity of $CHMO_{acinet}$ and its designed variants makes for ideal biocatalysts. Unfortunately, CHMO's practical application has been limited by drawbacks such as instability, requirement for NADPH as cofactor as well as by substrate and product inhibition. Improved variants through rational and semi-rational design has yielded mutants with increased oxidative and thermal stability [33–35]. Conversely, Reetz and co-workers reported on the directed evolution of phenylacetone monooxygenase (PAMO), a thermostable BVMO of which the wild-type enzyme does not accept cyclohexanone. Through several rounds of saturation mutagenesis, variants of PAMO were created that efficiently, albeit with much lower turnover frequency (TOF) than $CHMO_{actinet}$, converted cyclohexanone to ε-caprolactone with only a minimal trade-off in thermostability [36]. Recently, stable BVMOs able to accept cyclohexanone have been cloned and characterized from *Thermocrispum municipal* (TmCHMO) [37] and *Thermothelomyces thermophila* (PockeMO) [38]. Progress has also been made in improving $CHMO_{acinet}$'s ability to accept NADH as cofactor [39].

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In an attempt to find new BVMOs with diverse properties, we have been focusing on the BVMOs from *Aspergillus flavus*. Most of the cloned and characterized BVMOs are from bacterial origin, with fungal and other eukaryotic BVMOs only recently gaining interest [40–43]. We have previously reported on the cloning and characterization of four BVMOs from *A. flavus* [44,45]. This group of BVMOs were phylogenetically distinct from the CHMO-type subgroup and also display little to no activity towards simple cyclic ketones such as cyclohexanone. We have thus turned our attention to another four BVMOs of *A. flavus* that amongst the 26 predicted BVMOs in *A. flavus*, have the highest sequence similarity with CHMO.

2. Materials and methods

2.1. Phylogenetic analysis

Sequences were retrieved from the National Center for Biotechnology Information (NCBI). Multiple sequence alignments of the amino acid sequences were performed using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle) with the default parameters. An un-rooted maximum likelihood tree was constructed using MEGA 7 [46] using Nearest-Neighbour-Interchange (NNI) with bootstrap support for individual nodes calculated on 500 replicates. The Whelan and Goldman (WAG) model [47] was selected with a discrete Gamma distribution with five rate categories and by assuming that a certain fraction of sites are evolutionary invariable.

2.2. Cloning of BVMOs from A. flavus

The complete open reading frames of the BVMOs were PCR amplified from genomic DNA isolated from *A. flavus* NRRL3357. PCR reaction mixtures consisted of, to a final concentration, 1X KOD Hot Start Polymerase buffer, $MgSO_4$ (1.5 mM), deoxynucleoside triphosphates (0.2 mM each), KOD Hot Start DNA polymerase (0.02 $U.\mu l^{-1}$), gDNA (1 $ng.\mu l^{-1}$), and both the forward and the reverse primers (Table S1, Primer sets 1–4; 0.3 μM). The PCR was performed with an initial denaturing step at 95 °C for 2 min, followed by 18 cycles of denaturing at 95 °C (20 s), annealing at 55–58 °C (Table S1; 10 s), and elongation at 70 °C (1 min), with a final extension at 70 °C for 5 min. The amplicons were excised from an agarose gel and purified using a Biospin Gel Extraction Kit (BioFlux). Products were phosphorylated and ligated into pSMART (Lucigen) and transformed into *E. coli* TOP10 competent cells (Invitrogen), selected for on LB-plates containing 30 $\mu g.ml^{-1}$ kanamycin. Plasmid DNA was isolated from positive transformants using a Biospin plasmid DNA extraction kit (BioFlux) and inserts verified through restriction digestion and DNA sequencing. The ORFs were then sub-cloned in pET22b(+) (Novagen) using *Nde*I and *Xho*I, transformed and selected for on LB-plates containing ampicillin (100 $\mu g.ml^{-1}$).

The introns of $BVMO_{AFL334}$ were removed through sequential inverse PCR using the PCR protocol described above (Primer sets 5–7, Table S1), with the elongation step increased to 4 min. Following amplification, the template DNA was removed through *Dpn*I digestion. The purified amplicons were then circularized through phosphorylation and ligation. A similar strategy was used to create constructs for C-terminally Histidine tagged (CTH) proteins, by removing the stop codon and plasmid backbone between the cloned gene and the six histidine and stop codons encoded for by the plasmid (Primer sets 8–11, Table S1).

2.3. Heterologous expression and whole-cell biotransformations

The pET22:BVMO constructs were transformed into *E. coli* BL21-Gold(DE3) cells (Agilent Technologies) for heterologous expression. Expression was performed in auto-induction media (ZYP5052) containing 100 $mg.l^{-1}$ ampicillin at 20 °C for 48 h (200 rpm). Cells were harvested by centrifugation (7000 × *g*) and re-suspended as 0.1 g wet weight cells/ml 100 mM Tris-HCl (pH 8) containing 100 mM glucose and 100 mM glycerol. Biotransformations were performed in a reaction volume of 1 ml in capped 40 ml amber glass vials. Cyclohexanone was added to a final concentration of 10 mM [dissolved in methanol, final concentration in BRM = 1% (v/v)]. Reactions were performed at 20 °C with shaking (200 rpm) where after the reaction mixtures were extracted using an equal volume (2 × 0.5 ml) of ethyl acetate containing 2 mM 1-undecanol as an internal standard. GC-(-MS) analysis was performed with separation on a FactorFour VF–5 ms column (60 m × 0.32 mm × 0.25 μm, Varian). Protein expression was evaluated on SDS-PAGE using PageRuler Prestained Protein Ladder (ThermoScientific) and stained with Coomassie brilliant blue R-250.

2.4. Biotransformations and steady-state kinetics using purified BVMOs

For purification of the C-terminally histidine tagged (CTH) BVMOs, harvested cells were re-suspended as 0.2 g wet weight cells/ml buffer (50 mM Tris-HCl pH 7.4, 0.5 M NaCl, 40 mM imidazole). Cells were broken through a single passage in a continuous flow Benchtop Cell Disrupter (Constant Systems) at 30 kPsi. Cell-free extracts were obtained through centrifugation (7 000 × *g*, 15 min) and subsequent ultracentrifugation (100 000 × *g*, 90 min). The soluble proteins were loaded onto a 5 ml HisTrap FF Ni-affinity column (GE Healthcare) equilibrated in the same buffer. Unbound proteins were removed by washing with 10 column volumes of buffer, where after the BVMOs were eluted using a linear gradient of increasing imidazole concentration. Fractions containing the BVMOs were pooled and concentrated to approximately 2 ml through ultrafiltration (30 kDa NMWL, Amicon). Excess FAD was added to the proteins and left overnight. The proteins were afterwards desalted using size exclusion chromatography [Sephacryl S100HR gel-filtration column or PD-10 desalting columns (GE Helathcare)] with proteins eluted in 100 mM Tris-HCl buffer containing 100 mM NaCl. Protein concentrations were determined using the BCA protein assay (ThermoScientific).

Biotransformations using purified BVMOs were performed similar to the whole-cell biotransformations. Typical reaction mixtures consisted of 100 mM Tris-HCl buffer (pH 8) containing 2 μM BVMO, 1 U *Bm*GDH (purified *Bacillus megaterium* glucose dehydrogenase), 100 mM glucose, 0.3 mM $NADP^+$ and 10 mM substrate. Reactions were extracted after 2 h and 12 h with ethyl acetate containing either 2 mM 1-undecanol or 3-octanol as internal standard. GC(-MS) analysis was performed with separation on a FactorFour VF–5 ms column (60 m × 0.32 mm × 0.25 μm, Varian). Chiral analysis was performed with separation on a Chiraldex G-TA column (30 m × 0.25 mm × 0.12 μm, Astec). Conversions are based on relative GC areas of averaged duplicate experiments (standard deviations less than 9%).

Steady-state kinetics were performed by monitoring the oxidation of NADPH spectrophotometrically at 340 nm (20 °C; ε_{340nm} = 6.22 $mM^{-1}.cm^{-1}$). Reactions consisted of 100 mM Tris-HCl (pH 8), containing 100 mM NaCl, 0.3 μM purified enzyme, 0.3 mM NADPH and varying concentrations of cyclohexanone (1% (v/v) methanol as co-solvent).

3. Results and discussion

Our previous investigations into BVMOs from *Aspergillus flavus* focused on a phylogenetic subset of four BVMOs [44,45] grouped separately from well characterized BVMOs such as CHMO, PAMO, 4-hydroxy-acetophenone monooxygenase (HAPMO) [48] and cyclopentanone monooxygenase (CPMO) [49]. Despite their close phylogenetic relatedness to Group I BVMO which includes CPMO, none of these BVMOs, apart from $BVMO_{AFL210}$, showed conversion of most of the cyclic ketones tested. In order to identify fungal BVMOs with higher activity towards cyclic ketones, we turned our attention to another four BVMOs from *Aspergillus flavus* that amongst the 26 predicted BVMOs group closest to $CHMO_{acinet}$ or Group III BVMOs (Fig. 1). Of the four