Functional assembly of camphor converting two-component Baeyer-Villiger monooxygenases with a flavin reductase from *E. coli*

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Abstract

The major limitation in the synthetic application of two-component Baeyer-Villiger monooxygenases was addressed by identifying the 28 kDa flavin-reductase Fre from *E. coli* as a suitable supplier of reduced FMN for these enzymes. Coexpression of Fre with either 2,5- or 3,6-DKCMO from *P. putida* NCIMB 10007 significantly enhanced the conversion of camphor and norcamphor serving as representative ketones. With purified enzymes full conversion was achieved while only slight amounts of product were formed in the absence of this flavin reductase. Fusion of the genes of Fre and DKCMOs into single open reading frame constructs resulted in unstable proteins exhibiting flavin reducing, but poor oxygenating activity, which led to overall decreased conversion of camphor.

Keywords: Baeyer-Villiger monooxygenases, multi-component flavin-dependent monooxygenases, *Pseudomonas putida* NCIMB 10007, flavin reductase, Fre, fusion protein

Introduction

Chiral, but also achiral oxygenated and hydroxylated compounds are of great value, but their preparation through selective oxy-functionalisation of relevant substrates by conventional organic chemistry is difficult. For this reason there is an increasing interest in the application of biocatalysts to undertake these transformations (Bornscheuer et al. 2012). Specifically, enzymes were identified that catalyse the Baeyer-Villiger (BV) oxidation by incorporating one atom of molecular oxygen from air into a ketone substrate. This is advantageous compared to traditional chemical processes that are using environmentally harmful peracids (recent reviews: Balke et al. 2012; Leisch et al. 2011). The relevant enzymes, termed Baeyer-Villiger monooxygenases (BVMOs), are members of a superfamily of flavoprotein monooxygenases mechanistically related by their ability to activate molecular oxygen by generation of a covalent adduct with flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) (Harayama et al. 1992; Massey 1994; Walsh and Wencewicz 2013).

Flavin-dependent monooxygenases can be divided into subclasses according to structural criteria (van Berkel et al. 2006) enabling a distinction to be made between enzymes that combine the tasks arising from substrate oxygenation and flavin reduction within a single polypeptide chain, and enzymes that distribute the two tasks over separate functionally related proteins. Predominantly, enzymes that function as a single polypeptide (designated as Type I BVMOs: Willetts 1997) have been mostly investigated and are applied as biocatalysts in organic synthesis (de Gonzalo et al. 2010). Nowadays, a variety of Type I BVMOs are available and the historical prototype cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871, which was discovered and first purified almost 40 years ago (Donoghue et al. 1976), has been outranged by other candidates such as cyclododecanone monooxygenase (CDMO) from *Rhodococcus ruber* SC1 and CHMO from *Xantobacter* sp. XL5 with respect to their versatility and activity (Fink et al. 2012).

The structurally distinct two-component Type II BVMOs (Willetts 1997) should be considered together with other similar enzymes as members of a distinct group of multi-component flavin-dependent monooxygenases (MCMOs), which also includes bacterial luciferases with which they share high sequence homology, and various atypical hydroxylases (Ellis 2010). MCMOs are interesting enzymes since they exploit a loosely bound or freely diffusible reduced flavin as the ultimate source of reducing power to accept one atom of molecular oxygen not incorporated into the substrate in the concomitant biooxygenation reaction. This poses a major biochemical problem in aerobic cellular environments because reduced flavin cofactors undergo spontaneous non-selective one-electron reactions with triplet ground state molecular oxygen (Campbell and Baldwin 2009; Gibson and Hastings 1962). A number of MCMOs play important roles as biocatalysts in commercially important biotechnological applications. This includes the hydroxylase from *Streptomyces carbophilus* SANK 62585 employed for the bioconversion of mevastatin into the widely used hypocholestemic drug pravastatin (Matsuoka et al. 1989; Serizawa and Matsuoka 1991) and a number of luciferases exploited both directly and indirectly in various applications due to their characteristic bioluminescent properties (Hastings et al. 1985; Meighen 1991). Archetypal BV-oxidations have been recorded with a few Type II BVMO members of the MCMO family, such as the bacterial luciferases of *Photobacterium phosphoreum* NCIMB 844 and *Vibrio harveyii* ATCC 7744 (Villa and Willetts 1997; Willetts 1997), and 6-oxocineole monooxygenase of *Rhodococcus* sp. C1 (Williams et al. 1989). However, to date the best-characterised MCMO Type II BVMOs are two enantiocomplementary flavin-dependent isoenzymes involved in the metabolism of racemic camphor (**1**) by *Pseudomonas putida* NCIMB 10007 (= ATCC 17453). The relevant isoenzymes are 2,5- and 3,6-diketocamphane monooxygenase (2,5- and 3,6-DKCMO) that catalyse the biooxidation of the diketocamphane antipodes derived from (+)- or (–)-camphor to the corresponding lactones, which subsequently undergo spontaneous ring opening thereby triggering further catabolic

dissimilation (Conrad et al. 1965a; Jones et al. 1993; Taylor and Trudgill 1986). The two DKCMOs were recently cloned and recombinantly expressed (Kadow et al. 2011; 2012) but their exceptional catalytic potential was acknowledged much earlier in a number of studies applying whole cells or partly purified fractions obtained from the NCIMB 10007 strain (Gagnon et al. 1994; 1995; Grogan et al. 1993). Both isoenzymes operate *in situ* as loosely associated trimeric assemblages composed of the biooxygenating component plus a single flavin reductase (FR) (Jones et al. 1993). Genes encoding for both the enantio-complementary biooxygenating components (2 x 2,5-DKCMO, 1 x 3,6-DKCMO) are located on the linear 533 kb CAM plasmid (Iwaki et al. 2013) and are believed to have evolved from a single progenitor gene by the accepted mechanism of initial gene duplication and subsequent divergence (Horowitz 1945). The crystal structure of the monooxygenase subunit of 3,6-DKCMO has been published (McGhie et al. 1998). It is composed of a (β/α)8-barrel (TIM-barrel) with a hydrophobic core, which is a common motif among the flavin-dependent enzymes. The protein functions as a homodimer. No corresponding structure for the biooxygenating subunit of 2,5-DKCMO has been reported to date.

For many years it was accepted that the source of the reduced FMN for the biooxygenating components of each DKCMO isoenzyme was a monomeric NADH:FMN-dependent FR referred to variously as NADH:FMN oxidase (Conrad et al. 1965a; Trudgill et al. 1966) or NADH:FMN dehydrogenase (Taylor and Trudgill 1986). The molecular weight of this protein as assessed by its sedimentation characteristics has been reported consistently to be 36 kDa, although analysis of the amino acid complement of the purified protein indicated that the true MW was actually 43 kDa (Gunsalus et al. 1965); a figure consistent with extensive studies undertaken at the University of Exeter (Beecher 1997; Grogan 1995; McGhie 1998). Genetic exchange studies with the highly transmissible CAM plasmid (Rheinwald et al. 1973) led to the assumption that the gene coding for this FR, like those coding for both the DKCMOs, was located on the CAM plasmid, although extensive characterisation of this plasmid has failed

repeatedly to identify a relevant locus (Iwaki et al. 2013; Koga et al. 1989; Palchaudhuri and Chakrabarty 1976). It has been apparent for some time that the validity of a strict functional *in vivo* relationship between this particular FR and the biooxygenating subunits of the two DKCMO isoenzymes is open to question. Firstly, it has long been recognised that both trimeric assemblages are unstable (Conrad et al. 1965a; Taylor and Trudgill 1986) and preferentially use artificial electron acceptors such as 2,6-dichlorophenolindophenol (DCPIP) as effective sinks for transferred reducing power (Gunsalus et al. 1965); secondly, the equivalent purified FR components isolated from various MCMO luciferases sourced from bioluminescent bacteria are effective entities in promoting reducing power transfer to the biooxygenating subunits of both DKCMOs (Beecher 1997); and thirdly, various FRs with significantly different MWs (18.0 – 37.5 kDa) and relative reductive activities can be purified from preparations of the trimeric DKCMO isoenzymes obtained from cells harvested at different times throughout the logarithmic phase of growth of NCIMB 10007 on racemic camphor (McGhie 1998), reflecting similar growth-dependent variations in the nature of the equivalent FP subunits proposed for two other Type II BVMOs, 6-oxocineole monooxygenase from *Rhodococcus* sp. C1 (Williams et al. 1989) and the luciferase from *Vibrio harveyi* (Campbell and Baldwin 2009). Recently, two significant additional developments challenged further the long-standing proposed functional relationship of the trimeric DKCMOs first proposed by Gunsalus (Conrad et al. 1965a; Gunsalus et al. 1965) Initially, it was demonstrated that the genes encoding the biooxygenating subunits of both 2,5- and 3,6-DKCMO were functional when expressed in *E. coli* (Kadow et al. 2011; 2012). Subsequently, it has been shown that the biooxygenating components of each DKCMO isoenzyme perform efficient lactone-forming biooxygenation reactions in association with a homodimeric FR (2 x 18 kDa) encoded by the chromosomal DNA of NCIMB 10007 when tandem plasmid constructs carrying both relevant genes were expressed in *E. coli* (Iwaki et al. 2013).

In the light of these recent observations, the aim of the work reported here was: (i) to identify the FR enzyme(s) of *E. coli* responsible for the recently observed supply of reduced FMN to recombinant two-component BVMOs (Kadow et al. 2011; 2012) and (ii) to assemble any such competent functionality with the biooxygenating components of 2,5- and 3,6-DKCMO to promote their biooxygenating activity both via coexpression of the relevant genes (2,5-DKCMO x FR and 3,6-DKCMO x FR), and more ambitiously, via fusion of the relevant proteins into single open reading frame constructs (2,5-DKCMO-FR and 3,6-DKCMO-FR). The generation of Type II BVMO-based fusion proteins was considered to be particularly challenging, because although catalytically active fusion proteins created out of various Type I BVMOs together with phosphite dehydrogenase to achieve self-sufficient enzymes with respect to recycling the requisite nicotinamide cofactor have been reported (Torres Pazmiño et al. 2008, 2009), the relative complexity of successfully replacing the evolved substrate shuttle role of FMN in Type II BVMOs was likely to be more difficult to achieve.

Materials and Methods

Enzymes, chemicals and media

Phusion DNA polymerase and restriction enzymes were obtained from Thermo Fisher (Beverly, MA, USA). For SDS-PAGE the prestained PAGE ruler or PAGE ruler plus from Thermo Fisher was used. All other chemicals were purchased from Fluka (Buchs, Switzerland), Sigma-Aldrich (München, Germany) or Acros Organics (Geel, Belgium). Genomic DNA was isolated using the DNeasy Blood&Tissue Kit from Qiagen (Venlo, The Netherlands). Plasmid preparation, PCR purification and gel extraction kits were purchased from Promega (Madison WI, USA). HisTrap 5 mL and Sephadex G25 columns were obtained from GE Healthcare (Buc, France).

Multiple sequence alignment and sequence motif identification

Amino acid sequences of FMN reductase Lux G from *P. leognathi* (GenBank AAA25621), FMN reductase from *V. harveyi* (YP_001443581), NAD(P)H-flavin reductase from *V. fischeri* (YP_002158584), CDP-6-deoxy-delta-3,4-glucoseen reductase from *P. syringae* (EGH77205) and NAD(P)H-flavin reductase Fre from *V. fischeri* (BAA04596) were aligned using CLUSTALW (Larkin et al. 2007) and highly conserved regions were observed. This lead to the identification of the sequence motifs IDXPHGD/EXXL, PLLLIAXGTG and SILD/EXC that were used to search for within the genomic sequence of *E. coli*, which was initially translated into all possible translational frames. Putative open reading frames (ORFs) containing these motifs were subjected to homology search using BlastX.

Amplification and cloning

Amplification of the entire Fre gene, including the 12 N-terminal amino acids MPYLSIRQRKR additional to the published sequence (GenBank: M61182), from genomic DNA of *E. coli* BL21 (DE3) was performed with primers with the restriction sites for *Nde*I

and *Bam*HI for cloning into the plasmid pGaston (pGas, Table 1). After initial denaturation for 5 min at 95 °C, the cycling program was followed for 5 cycles: 45 s 95 °C denaturation, 45 s 53.5 °C primer annealing, 70 s 72 °C elongation and then for 20 cycles: 45 s 95 °C denaturation, 45 s 48.5 °C primer annealing, 70 s 72 °C elongation. The final elongation step lasted over 10 min at 72 °C. PCR-amplificates were digested with *Nde*I and *Bam*HI and ligated into pGas digested with the same enzymes. The resulting plasmid contained the respective reductase fused to a C-terminal His-tag, which was confirmed by DNA-sequencing.

Fusion proteins composed of either Fre and a DKCMO were constructed by amplification of the particular genes, assembling them to large fragments by overlap extension PCR and subsequent cloning. Therefore, genes were amplified with primers encoding a restriction site at the end that would later be linked with the plasmid (Table 1), and the sequence of the desired linker on the end pointing towards the fusion site. Sticky ends containing the linker sequence were thus created and subsequently assembled in a PCR with the following program: 98 °C for 30 s, then 16 cycles of 98 °C for 10 s, 68 °C (−0.5 °C per cycle) for 30 s, 72 °C for 1 min. The program was continued with 98 °C for 30 s, then 16 cycles of 98 °C for 30 s, 68 °C (−0.5 per cycle) for 30 s, 72 °C for 1 min, and then a final elongation of 72 °C for 10 min after addition of primers for cloning into the desired plasmid. The final fragments were purified via agarose gel electrophoresis and digested with restriction enzymes (Table 1). Digested fusion genes were ligated with the respective equally digested target plasmid using T4 DNA-ligase. *E. coli* DH5α (ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 end A1 gyrA96 thi-1 relA1) was transformed with ligation products and propriety of the obtained constructs was confirmed by DNA-sequencing. All fusion proteins were composed of the Fre variant that contained the additional 12 amino acids MPYLSIRQRKR at the N-terminus compared to the published sequence (GenBank: M61182). Additionally, fusion protein E, F as well as H_6F

variants using the shorter sequence were constructed. Those did not show altered properties compared to the longer versions.

Bacterial strains and culture conditions

Transformation of *E. coli* strain BL21 (DE3) (Novagen, genotype: [95 F– ompT hsdSB (rB-mB-) gal dcmrne131 (DE3)]) was carried out by the heat shock method described by Chung *et al*. (1989). *E. coli* cells expressing Fre were cultivated in Luria Bertani (LB) medium (10 g tryptone, 5 g yeast, 5 g NaCl in 1 L dest. H_2O) supplemented with 50 µg/mL carbenicillin. Cultures were inoculated from an overnight culture and grown to an OD_{600} of 0.5 at 37 °C. Induction was performed with L-rhamnose in a final concentration of 0.2%. Cells were harvested after 16 h of cultivation at room temperature (RT, 20-25 °C) and 200 rpm. Expression of 2,5- and 3,6-DKCMO was performed as described elsewhere (Kadow et al. 2011; 2012). Coexpression of Fre together with a DKCMO was achieved in terrific broth (TB) medium supplemented with kanamycin and carbenicillin plus chloramphenicol in case of coexpression of chaperones GroES/GroEL. Expression of all proteins simultaneously was induced at an OD_{600} of 0.5 by addition of 0.2 mM IPTG, 0.2% (w/v) L-rhamnose and 0.2% (w/v) arabinose, if required for chaperone expression. Cultivation was continued for 16 h at RT and 200 rpm. Fusion gene expression was performed as described above with the exception that LB-medium was used for expression of fusion protein A and F.

Gene expression analysis

Gene expression analysis was performed with crude cell extract. Samples standardized to an OD_{600} of 7 were taken during cultivation. Cells were harvested by centrifugation and resuspended in sodium phosphate buffer (100 mM, pH 7.5) containing 300 mM sodium chloride. Cell disruption was performed with a MiniLys device (1 min at 4 rpm; Bertin, Montigny-le-Bretonneux, France). For SDS-PAGE analysis, the supernatant was substituted

with Laemmli buffer (Laemmli 1970). SDS-PAGE was carried out on 10-12% resolving gels. Proteins were stained with a Coomassie R250/G250 solution.

Enzyme purification

Cells were harvested by centrifugation and resuspended in sodium phosphate buffer (100 mM, 300 mM NaCl, pH 7.5). Cell disruption was performed as described above. Recombinant enzymes were purified by affinity chromatography via His-tag on an automated Äkta purifier system. After centrifugation of disrupted cells for 1 h at 10,000 x g, the supernatant was filtered and added to a 5 mL HisTrap column with bound Ni^{2+} that was equilibrated with sodium phosphate buffer (100 mM, pH 7.5) supplemented with 300 mM NaCl. After passing through of the crude extract, the column was washed with three column volumes of sodium phosphate buffer (100 mM, pH 7.5) supplemented with 300 mM NaCl and 15 mM imidazole followed by two column volumes of sodium phosphate buffer (100 mM, pH 7.5) supplemented with 300 mM NaCl and 30 mM imidazole to remove non-specifically bound proteins. Elution was performed by adding three column volumes of 500 mM imidazole in sodium phosphate buffer (100 mM, pH 7.5) supplemented with 300 mM NaCl. Fractions of washing and elution steps were collected to analyse purity by SDS-PAGE. In order to remove imidazole and NaCl from the eluate, the pooled elution fractions were loaded to two 5 mL size exclusion column (Sephadex G25 matrix) connected in series, which were equilibrated with sodium phosphate buffer (100 mM, pH 7.5) before. Protein fractions were detected via online absorption measurement at 280 nm and collected. Determination of protein content was carried out with the Bradford method and a standard curve of BSA in the same buffer in a range of 0.75 – 0.006 mg/mL was used (Bradford 1976).

Determination of flavin reductase activity

Determination of FR activity was performed by following the consumption of NADH via measuring the decrease in absorption at 340 nm over 30 min in 200 µL scale. Pure enzymes

were used in concentrations between 20 and 100 µg/mL. Specific activity is given in units per milligram (U/mg) protein. One unit is defined as the amount of enzyme that catalyses the reduction of 1 µmol FMN per minute.

Determination of kinetic properties of fusion proteins

For determination of flavin reduction kinetics of fusion proteins, the concentration of FMN was varied between 0.5-500 µM. Pure fusion protein concentrations were varied between 25-100 µg/mL. Experiments were perfomed in triplicates revealing measurement values with a standard deviation of 9% on average. From those the average was calculated to estimate K_M and V_{max} from the linear dependence (R^2 = 0.992) of [FMN] vs. [FMN] x initial activity (U/mg) in a Hanes-Woolf plot (Hanes 1932).

Biocatalytic reactions and GC analysis

For biocatalysis, whole-cell preparations of *E. coli* BL21 overexpression cultures as well as pure enzymes were applied. Reactions were carried out in sodium phosphate buffer (100 mM, pH 7.5). Ketone substrate concentration was 2 mM. In whole-cell biocatalysis, cells were resuspended in buffer supplemented with 2% glucose (w/v) after harvest to achieve an OD_{600} of 20-25. No additional cofactors were added. Pure Fre as well as DKCMOs were employed in concentrations of 1-2 mg/mL, pure fusion enzymes were standardized to 0.5 mg/mL. In these reactions FMN was added to a final concentration of 0.3 mM and NADH was used in a concentration of 0.4 mM. 2 U/mL formate dehydrogenase from *Candida biodinii* (FDH) were added together with 10 mM sodium formate. Incubation was performed in 24-well MTP at RT and 800 rpm. Sample volume was 500 µL. Conversion was determined by quantification of product formation via GC. For quantification, standard curves of substrates with concentrations of 0.5-10 mM were measured. Ethylbenzoate was used as an internal standard. All experiments were performed in duplicates.

Extraction of samples was performed by vortexing of samples with 500 µL of ethyl acetate. Separation of aqueous and organic phase was achieved by centrifugation. Samples were dried over anhydrous sodium sulphate and analysed on a Varian 3800 gas chromatograph applying a 25 m 0.25 µM CP-Chirasil-Dex CP capillary column (Varian, Middelburg, The Netherlands). Injection temperature was set to 250 °C. Column temperature for (+)-1, (–)-1 and norcamphor was 80 °C for 5 min followed by a gradient of 20 °C/min to 90 °C and then 3 °C/min to 120 °C. This was maintained for 5 min and then followed by heating the column to 200 °C with 4 °C/min which was hold for 3 min.

Results

Identification of flavin reductase Fre from *E. coli*

To fully exploit the FMN-reducing activity of *E. coli* to provide reduced FMN for the DKCMOs, it was necessary to identify, clone and functionally express the responsible enzyme to verify and modulate its interaction with the DKCMOs.

It was supposed that it would be a flavin reductase (FR) with properties similar to other FR-elements of known two-component monooxygenase systems. From a multiple sequence alignment of several such representatives three specific protein sequence motifs were derived and subsequently applied to map similar genes in the genome sequence of *E. coli* BL21 (GenBank: NC_102971.2, see materials and methods for details). Only one reasonable ORF was identified that corresponds to a putative protein containing any of these motifs and that ORF actually contained single copies of all three. A homology search of the translated sequence yielded highest homology to a 28 kDa protein already annotated as putative FMN-reductase. Despite the fact that the identified ORF contains twelve additional N-terminal amino acids, the gene corresponds to the FR of *E. coli* that had already been identified in 1987 as a reductase involved in the activation of ribonucleotide reductase by supplying reduced flavin for the reduction of the Fe^{3+}-centre of this enzyme to Fe^{2+} (Fontecave et al. 1987). The enzyme was named Fre and the gene was cloned in 1991 (GenBank: M61182), but its recombinant expression in *E. coli* JM 109 was not efficient (Spyrou et al. 1991). In this work, Fre was expressed from the plasmid pGAS in BL21 (DE3), which led to a high amount of soluble protein (data not shown).

Functional combination of Fre from *E. coli* with the DKCMOs

Initial experiments to study the interaction of recombinantly produced Fre with the DKCMOs were first carried out by combination of crude cell extracts that contain the separately expressed enzyme activities. However, this strategy turned out to be inefficient due to the

requirement of constant supply with NADH. To circumvent this problem, we utilized the natural NADH-recycling machinery of *E. coli* by applying resting cells as catalysts. Therefore it was necessary to coexpress both Fre and either of the two DKCMOs in the same host. Two different plasmids providing different antibiotic resistances and promoters for induction of protein synthesis were used to produce the components individually, which made it unproblematic to coexpress both proteins using these plasmids in the same *E. coli* host. By varying the time points of induction, temperature and the culture medium, it was concluded that the best coexpression was achieved when the required genes were induced simultaneously at OD_{600} 0.5 followed by cultivation at RT for 16 h in TB-medium. The chaperones GroES/GroEL that were applied earlier in 3,6-DKCMO expression (Kadow et al. 2012) were used when Fre was coexpressed as well, leading to a high amount of soluble protein (data not shown).

When resting cell preparations containing Fre coexpressed together with 2,5-DKCMO were applied in biooxidations of either camphor enantiomer as well as norcamphor as model substrates using a substrate concentration of 2 mM, almost full conversion (96%) of the favoured (+)-enantiomer of camphor was achieved after 1.5 h (Figure 1). In comparison, 74% were achieved after 4 h when only 2,5-DKCMO was overexpressed. Here, one must keep in mind, that the natural basal expression of Fre is still taking place in the *E. coli* cells enabling background oxidation of the substrate. Moreover, 39% conversion of norcamphor after 4 h was obtained when Fre and 2,5-DKCMO were simultaneously overexpressed, compared to 18% of formed product when only 2,5-DKCMO was recombinantly produced by the cells. Applying cells coexpressing Fre together with 3,6-DKCMO gave 77% conversion of (–)-camphor after 1.5 h compared to only 14% when Fre was not coexpressed. Although it was repeatedly reported (Iwaki et al. 2013) that both DKCMO isoenzymes would exhibit absolute specifity for the respective camphor enantiomer that is the precursor for their physiological substrate, very small amount of product formation from the opposite camphor enantiomer

were observed in both cases (Figure 1), data consistent with earlier studies untertaken with these purified enzymes (Beecher 1997; Grogan 1995).

An alternative strategy of keeping the level of available NADH constant is to add a cofactor-recycling enzyme (Figure 2). A frequently applied recycling enzyme is formate dehydrogenase from *Candida boidinii* (FDH) that has the advantage of accepting sodium formate as cheap substrate and pushing the equilibrium towards NADH-formation by production of carbon dioxide (Bommarius and Drauz 1994). Applying this enzyme contributed to fully unveil the beneficial effect of Fre from *E. coli* using purified DKCMOs (Figure 3). Biocatalytic studies with the model substrates mentioned above were undertaken using individually expressed and affinity-purified DKCMOs and purified Fre, with addition of FDH and sodium formate to the reaction mixture. The conversion of (+)-camphor catalysed by pure 2,5-DKCMO was >99% after 4h hours and about the same conversion was observed in the reaction of (–)-camphor with pure 3,6-DKCMO after only 1.5 h. Without addition of Fre only 4% of products derived from the respective camphor enantiomers were detected. Norcamphor was converted to 69% by pure 2,5-DKCMO and to 32% by pure 3,6-DKCMO when the reaction contained purified Fre, but only slight amounts of product were found in the absence of Fre. The lower activity of this substrate compared to the substituted bornanone camphor is in agreement with equivalent data obtained by Iwaki *et al.* (2013).

Creation of various fusion proteins

The two-component character of the DKCMOs and other MCMOs is associated with the disadvantage of requiring the production and purification of two distinct proteins. To overcome these drawbacks it was our aim to fuse the two components together to form a continuous polypeptide that features both catalytic activities. Different approaches to achieve such protein fusions have been described and most approaches made use of a linker-sequence that was added in-between the two enzymes to ensure adequate subunit flexibility. Two

different linkers were applied in this work. While the first one (L1) is the ten amino acid linker SSGLVPRGSH that is used as a standard feature in pET-vectors to fuse the target protein with a His-tag, the second linker (L2) consists of the twelve amino acids GTSGSSGSGGGG that were shown to effectively fuse the identical subunits of the single-chain Arc repressor (Robinson and Sauer 1998). Furthermore, the orientation of the relevant genes encoding Fre and the respective DKCMO were varied, since it was not known on which term of a specific subunit the addition of a second protein would have least influence on its activity. Finally, the location of the His-tag was varied between the N- and C-terminus. The exact structure of every investigated fusion enzyme is depicted in Table 2. Thus, seven different variants were created and investigated. A major problem was the reduced stability of all candidates. Bands with the size of 70 kDa were mainly observed in the insoluble fraction although a variety of expression conditions were tested, while decomposition in the supernatant was observed in all cases. After affinity purification and desalting of the fusion proteins, mainly fragments shorter than the expected 70 kDa were observed (data not shown). When tested, all of the purified fusion proteins were active in the reduction of FMN by NADH. V_{max} of all pure constructs was in the range of 0.1 U/mg and K_M for FMN is app. 10 µM (Table 2). Unfortunately, no or only moderate monooxygenation of camphor was observed with the fusion enzymes. In the best case of fusion enzyme B, 15% product formation was detected when (–)-camphor was the substrate (Table 2). Although the orientation of the distinct subunits in fusion enzymes E and F are similar to that in fusion enzyme B, no camphor conversion was observed for these variants, which might be due to adverse effects of L2 on the protein. Moreover, pure fusion enzyme G, in which the orientation of Fre and 2,5-DKCMO was reversed did not convert camphor at all. Reasonable oxidation of camphor was not observed applying variants that contained a N-terminal His-tag.

Discussion

The dual opportunity to perform Baeyer-Villiger oxidation applying oxygen as the oxidative reactant and deliver excellent regio- and/or enantioselectivity has been a driving force for the surge in interest on BVMOs during the last two decades. However, while a variety of single-component Type I BVMOs have been discovered, characterised, and in some cases targeted for successful protein engineering to the extent that they are now becoming applied in industrial processes (Balke et al. 2012; Leisch et al. 2011), relevant multi-component (Type II) BVMOs such as the isoenzymic DKCMOs from *P. putida* NCIMB 10007, have been relatively neglected so far even though their powerful catalytic qualities have been recognised for almost 20 years (Willetts 1997). An important drawback in the application of MCMOs has been the requirement for a FR-type protein that can serve as a robust effective shuttle for the requisite reduced FMN. During our studies on the recombinant production of both DKCMOs from NCIMB 10007 (Kadow et al. 2011; 2012) we discovered that an enzyme activity in *E. coli* was apparently fulfilling this key shuttle role for these Type II BVMOs. Hence, it became our further objective to identify the responsible enzyme, optimise its effectiveness, and thereby increase the attractiveness of DKCMOs as useful biocatalysts. The active FR Fre in *E. coli* was identified because of its sequence homology with other FRs that promote a sequential mechanism of hydride transfer from a nicotinamide to a flavin cofactor. Fre is a 26 kDa monomeric protein composed of 232 amino acid residues, which was first discovered and characterised in 1987 (Fontecave et al. 1987). A sequential mechanism is a characteristic mainly described for candidate enzymes that do not retain the flavin tightly bound, but use it as a substrate (Ellis 2010). Accordingly Fre, which is able to use loosely bound riboflavin, FMN or FAD (in that order of preference) as a substrate, is not a true flavoprotein. The versatility of Fre is not restricted to the flavin cofactor specificity but it can transfer reducing power from either NADPH or NADH, which is unusual for an

oxidoreductase. The monomeric nature of Fre is a feature shared with the FMN-dependent FR partner of the EDTA degrading monooxygenase EmoA (Payne et al. 1998) and distinguishes these enzymes from the FMN reductases of other MCMOs that are characteristically homodimeric (Ellis 2010). The crystal structure of Fre was published in 1999, revealing two principal domains, a N-terminal flavin-binding domain and a C-terminal domain with an α/β-fold believed to be responsible for NAD(P)H binding (Ingelman et al. 1999). In this respect, Fre shows considerable general similarity to the ferrodoxin-$NAD(P)^+$-reductase family (FNR), although overall sequence homology of Fre to these enzymes is not significant (Nivière et al. 1996). Although Fre-like enzymes are known to be widely distributed in luminous bacteria which are characterised by the presence of a MCMO luciferase, they apparently do not serve as the major active partners of these Type II BVMOs. This physiological role is fulfilled on a species-specific basis either by LuxG (an equivalent FR that operates with unbound FMN; (Nijvipakul et al. 2008) or alternatively FRase 1 or FRP, both different types of FR that operate with tightly-bound FMN (Zenno and Saigo 1994).

High levels of lactonisation of both camphor antipodes obtained from assemblages of purified Fre and the relevant pure DKCMO oxygenating component from NCIMB 10007 confirmed that Fre, although not the only potential FR activity present in *E. coli* capable of delivering reduced FMN, is a potent coupling partner for these particular MCMOs. This finding is interesting because its reported sequential mechanism differs from the ping-pong mechanism of the MCMOs in *Vibrio harveyi* (luciferase + FRP) and *Vibrio fischeri* (luciferase + FRase 1; Ellis 2010). A similar sequential reaction mechanism was indicated by the kinetic characteristics of Fred from *P. putida* NCIMB 10007, a NADH:FMN-dependent FR that was recently shown to be able to support BV-activity in reconstructed DKCMO isoenzyme assemblages (Iwaki et al. 2013). Fred, like the majority of other FRs that support MCMOs, is homodimeric in its functional state (2 x 18 kDa). This enzyme differs from the DKCMO-supporting monomeric 36 kDa NADH:FMN dehydrogenase assigned that role in

NCIMB 10007 by earlier studies (Conrad et al. 1965a; Conrad et al. 1965b; Taylor and Trudgill 1986).

These data for DKCMO assemblages from NCIMB 10007, which confirm that these two-component monooxygenases can functionally interact with different FRs, even crossing species boundaries, complement previous results obtained with another Type II BVMO, the 6-oxocineole monooxygenase from *Rhodococcus* sp. C1 (Williams et al. 1989). Altogether, the data support the proposal that for successful growth of these microorganisms, the crucial parameter at any particular time *in vivo* may be the total amount of reducing equivalents and that such enzymes, rather than relying on a single dedicated FR as a source of reducing power, exploit their relaxed specificities as a way of capitalising on the most abundant source available. This conclusion is supported by the observation that various FRs with significantly different MWs (18.0 – 37.5 kDa) and relative reductive activities can be purified from preparations of the trimeric DKCMO isoenzymes sourced from cells harvested at different times throughout the logarithmic phase of growth of NCIMB 10007 on racemic camphor (McGhie 1998). Besides Type II BVMOs, several other MCMOs have been shown to exhibit relaxed specificity with respect to the predisposed requirement for a source of reduced flavin cofactor. For instance, the gene coding for the biooxygenating subunit of the hydroxylase from *S. carbophilus* SANK 62585 is highly active when expressed in various alternative host microorganisms, presumably exploiting one or more host-coded FRs (Klaassen et al. 2010). Similarly, *lux*AB reporter gene cassettes sourced from various bioluminescent donors function in a wide range of host organisms, again exploiting one or more FR activities of the hosts (Hastings et al. 1985; Meighen 1991). Another reflexion of this relaxed specificity is the preparation of highly active 'fused' flavin monooxygenases that has been reported. In this context it is important to mention the *lux*AB plus FR concatenated monocistronic operon constructed from the relevant genes of *Vibrio harveyii* (Jawanda et al. 2007). Moreover the hybrid-fused enzyme constructed from the biooxygenating component of the mevastatin

hydroxylase from *S. carbophilus* SANK 62585 and a FR-coding region from the self-sufficient P450RhF monooxygenase from *Rhodococcus* sp. NCIMB 9784 (Nodate et al. 2006) were described (Klaassen et al. 2010).

Inspired by these cited examples, and the previous reports of successful Type I BVMO-based fusion proteins (Torres Pazmiño et al, 2008, 2998), attempts were made in this study to create functional fusions of Fre and the oxygenating component of either 2,5- or 3,6-DKCMO within one single protein using two different linkers and two different relative orientations of the FR and 2,5- or 3,6-DKCMO oxygenating component genes to generate several different organisational variants (Table 2). While the FR activity of all seven investigated fusion proteins was not influenced significantly by being concatenated with either of the larger oxygenating subunits, all of the tested combinations exhibited a substantial detrimental effect on the activity of the monooxygenase protein moiety, and considerable decomposition was observed in all cases. Possible reasons for this poor overall level of performance could be either structural, in the sense that the constructs used represent dimeric assemblages, whereas *in vivo* it is known that both DKCMOs operate as trimeric assemblages, or functional in the sense that by fusing the FR activity to the oxygenating protein this in some way prevents the significant domain movements known to accompany the mode of action of BVMOs (Mirza et al. 2009). Although one fusion enzyme was created (Fus B) that exhibited both FR- and some limited 3,6-DKCMO-related oxidation activity, higher conversions were achieved by either applying pure, separately produced enzymes or resting cells overexpressing both proteins. These two systems have been shown to be powerful and unelaborate solutions facilitating the actual applicability of recombinant DKCMOs.

It remains to be established whether successful lactone-forming fusion proteins based on alternative two-gene constructs can be generated from a DKCMO isoenzyme oxygenating component and either the NCIMB 10007 encoded Fred or another of the genes corresponding to any of the alternative competent FRs recently identified in NCIMB 10007 (McGhie 1998).

More ambitiously, more complex fusion proteins based on trimeric constructs reflecting the trimeric structures of the native DKCMOs in NCIMB 10007, and equivalent to the successful *lux*AB plus FR concatenated monocistronic operon constructed from the relevant genes of *Vibrio harveyii* (Jawanda et al. 2007) should be investigated.

When undertaking any monooxygenase-dependent biotransformation, the availability of stoichiometric amounts of the relevant reduced nicotinamide coenzyme is essential to optimise product formation. Unlike with Type I BVMOs, which are characterised by deploying tightly-bound FAD to transfer reducing power from NADPH to the active site of the enzyme, the problem is further exacerbated for loosely assembled MCMOs such as the Type II BVMOs because of the susceptibility of unbound and loosely bound reduced FMN to undergo spontaneous reactions with molecular oxygen, thus dictating that an adequate supply can only be guaranteed when it is constantly produced (Gibson and Hastings 1962).

These caveats were initially illustrated in this study by comparing the poor outcomes achieved by combining together crude cell extracts of *E. coli* that contain either a separately expressed recombinantly produced Fre or a DKCMO isoenzyme oxygenating subunit (data not shown), with the much more efficient equivalent outcomes achieved by exploiting the natural NADH-recycling machinery of *E. coli* by deploying resting cell preparations containing both recombinant components (Figure 1). The stoichiometric efficiency of lactone production by the DKCMOs could be enhanced even further by combining affinity-purified DKCMOs and Fre with an appropriate coenzyme recycling system promoted by the addition of FDH from *C. boidinii* (Figure 3).

Overall, our work displays valuable evidence, that MCMOs exhibit relaxed specificity with respect to the exploited source of reduced flavin possibly related to the susceptibility of unbound reduced cofactor to undergo spontaneous oxidation resulting in an evolved need to source reduced flavin cofactors from wherever it is most readily available at any given time in aerobic cellular environments.

Authors' contributions

M.K. and U.T.B. initiated the project and designed the overall strategy. M.K. and K.B. performed the experimental work. All authors cowrote the paper.

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Figure legends

Fig. 1 Comparison of camphor and norcamphor conversion by resting *E. coli* cells overexpressing both, a DKCMO and Fre compared to cells overexpressing only a DKCMO. a) I: Overexpression of only 2,5-DKCMO, II: Coexpression of 2,5-DKCMO and Fre. b) I: Overexpression of only 3,6-DKCMO, II: Coexpression of 3,6-DKCMO and Fre. Substrate concentration was 2 mM. NADH-recycling was accomplished by the machinery of the cells. Conversion was determined by quantification of product formation via GC.

Fig. 2 Combined system of two-component Baeyer-Villiger monooxygenase 2,5-DKCMO and flavin reductase Fre from *E. coli*. The monooxygenase is supplied with reduced FMN by the NADH-consuming Fre. This cofactor is simultaneously recycled using formate dehydrogenase from *C. boidinii.*

Fig. 3 Comparison of camphor and norcamphor conversion by pure DKCMOs combined with pure Fre with obtained conversions when only the DKCMO was used. a) I: 2,5-DKCMO, II: Combination of 2,5-DKCMO and Fre. b) I: 3,6-DKCMO, II: Combination of 3,6-DKCMO and Fre. Substrate concentration was 2 mM. Recycling of NADH was accomplished by addition of FDH and sodium formate. Conversion was determined by quantification of product formation via GC.

Tables

Table 1 List of oligonucleotides applied in this work

Name	**Purpose**	**Sequence (5'- to 3')**[l]
*Nde*I_Fre-fw	Amplification of Fre for cloning into pGas	GGAATTCCATATGCCCTATTTATCGATCCGACAGAG
Fre_*Bam*HI-rv		CGCGGATCCGATAAATGCAAACGCATCGCCA
*Nco*I_2,5-DKCMO-fw	Amplification of 2,5-DKCMO for fusion genes A and E	CATGCCATGGTGAAATGCGGATTTTTCCATACCCCA
2,5-DKCMO_L1-rv		CGCGGCACCAGGCCGCTGCTGCCCATTCGAACCTTCGGTAAC
2,5-DKCMO_L2-rv		CCGCTGCTGCCGCTGGTGCCGCCCATTCGAACCTTCGGTAAC
*Nde*I_3,6-DKCMO-fw	Amplification of 3,6-DKCMO for fusion genes B and F	GGAATTCCATATGGCAATGGAAACTGGTTTGATCT
3,6-DKCMO_L1_rv		CCAGGCCGCTGCTACGCTTAGGCAGGAGAATCTTTGG
3,6-DKCMO_L2-rv		CCGCTGCTGCCGCTGGTGCCACGCTTAGGCAGGAGAATCTTTGG
L1_Fre-fw	Amplification of Fre for fusion genes A, B, E and F	AGCAGCGGCCTGGTGCCGCGCGGCAGCCATCCCTATTTATCGATCCGACAGAGAAAGC
L2_Fre-fw		GGCACCAGCGGCAGCAGCGGCAGCGGCAGCGGCGGCAGCGGCAGCGGCGGCGGCATGCCCTATTTATCGATCCGACAGAG
Fre_*Xho*I_rv	Amplification of Fre for fusion genes A and E	CCGCTCGAGGATAAATGCAAACGCATCGCCA
Fre_STOP_*Bam*HI-rv	Amplification of Fre for fusion genes H_6B and H_6F	CGCGGATCCTCAGATAAATGCAAACGCATCGCCA
Fre_L2-rv	Amplification of Fre for fusion gene G	GCCGCCGCCGCCGCTGCCGCTGCCGCCGCTGCCGCTGCCGCTGCTGCCGCTGGTGCCGATAAATGCAAACGCATCGCCA
L2_2,5-DKCMO-fw	Amplification of 2,5-DKCMO for fusion gene G	GGCAGCGGCGGCGGCGGCAAATGCGGATTTTTCCATACCCCATACA
2,5-DKCMO_*Xho*I-rv		CCGCTCGAGTTAGCCCATTCGAACCTTCGGTAAC

Table 2 Overview of properties of fusion proteins constructed of Fre and the DKCMOs

Fusion Protein	Organisation	Plasmid	Linker	His-tag position	Vmax[a] [U/mg]	K_M[a] [µM]	Camphor-conversion (%)	
							Resting cells[b]	Pure enzymes
A	2,5-DKCMO_Fre	pET28	L1	C-terminal	0.194	7	2 ± 0[c]	10[c]
B	3,6-DKCMO_Fre	pGas	L1	C-terminal	0.338	2.4	4 ± 0[d]	15 ± 0[d]
H_6B	3,6-DKCMO_Fre	pET28	L1	N-terminal	0.149	8.3	2 ± 0	–
E	2,5-DKCMO_Fre	pET28	L2	C-terminal	0.130	2.5	–	–
F	3,6-DKCMO_Fre	pGas	L2	C-terminal	0.202	8.4	–	–
H_6F	3,6-DKCMO_Fre	pET28	L2	N-terminal	0.171	19.1	6 ± 1[d]	3 ± 2[d]
G	Fre_2,5-DKCMO	pET28	L2	C-terminal	0.196	7.0	5 ± 1[c]	–

Linker 1: SSGLVPRGSH. Linker 2: GTSGSSGSGGGG

[a] Pure fusion proteins were applied to determine kinetic data; [b] resting cells that were expressing fusion proteins were used; [c] 2 mM (+)-camphor was used as the substrate; [d] 2 mM (–)-camphor was used as the substrate

Figures

Figure 1

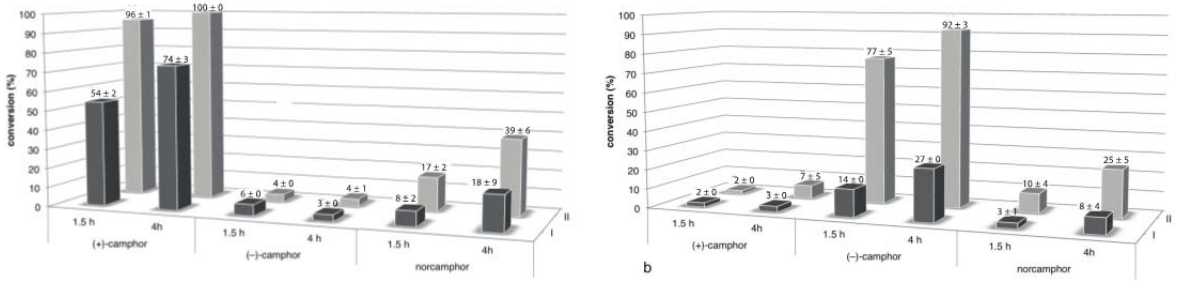


Figure 2

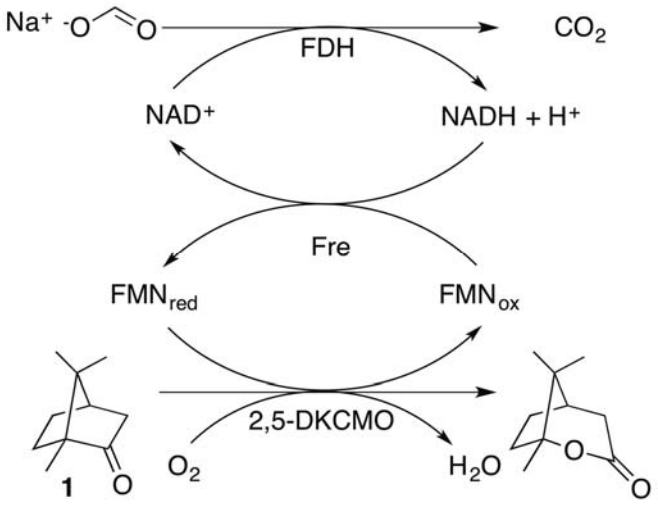
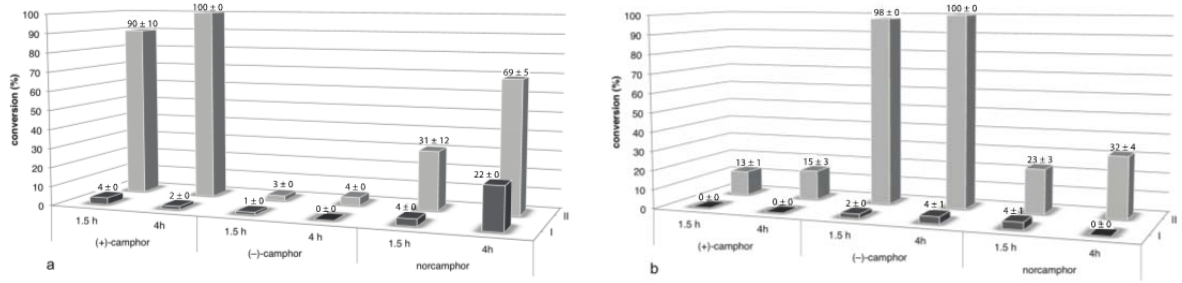


Figure 3


conversion (%)
100
90
80
70
60
50
40
30
20
10
0
90 ± 10
100 ± 0
69 ± 5
31 ± 12
22 ± 0
4 ± 0
2 ± 0
1 ± 0
3 ± 0
0 ± 0
4 ± 0
4 ± 0
1.5 h
4h
1.5 h
4 h
1.5 h
4h
(+)-camphor
(–)-camphor
norcamphor
a
II
I
conversion (%)
98 ± 0
100 ± 0
13 ± 1
15 ± 3
23 ± 3
32 ± 4
0 ± 0
0 ± 0
2 ± 0
4 ± 1
4 ± 1
0 ± 0
1.5 h
4h
1.5 h
4h
1.5 h
4h
(+)-camphor
(–)-camphor
norcamphor
b
II
I