

Camphor Pathway Redux: Functional Recombinant Expression of 2,5- and 3,6-Diketocamphane Monooxygenases of *Pseudomonas putida* ATCC 17453 with Their Cognate Flavin Reductase Catalyzing Baeyer-Villiger Reactions

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Whereas the biochemical properties of the monooxygenase components that catalyze the oxidation of 2,5-diketocamphane and 3,6-diketocamphane (2,5-DKCMO and 3,6-DKCMO, respectively) in the initial catabolic steps of (+) and (−) isomeric forms of camphor (CAM) metabolism in *Pseudomonas putida* ATCC 17453 are relatively well characterized, the actual identity of the flavin reductase (Fred) component that provides the reduced flavin to the oxygenases has hitherto been ill defined. In this study, a 37-kDa Fred was purified from a camphor-induced culture of *P. putida* ATCC 17453 and this facilitated cloning and characterization of the requisite protein. The active Fred is a homodimer with a subunit molecular weight of 18,000 that uses NADH as an electron donor ($K_m = 32 \mu\text{M}$), and it catalyzes the reduction of flavin mononucleotide (FMN) ($K_m = 3.6 \mu\text{M}$; $k_{\text{cat}} = 283 \text{ s}^{-1}$) in preference to flavin adenine dinucleotide (FAD) ($K_m = 19 \mu\text{M}$; $k_{\text{cat}} = 128 \text{ s}^{-1}$). Sequence determination of ~40 kb of the CAM degradation plasmid revealed the locations of two isofunctional 2,5-DKCMO genes (*camE*₂₅₋₁ for 2,5-DKCMO-1 and *camE*₂₅₋₂ for 2,5-DKCMO-2) as well as that of a 3,6-DKCMO-encoding gene (*camE*₃₆). In addition, by pulsed-field gel electrophoresis, the CAM plasmid was established to be linear and ~533 kb in length. To enable functional assessment of the two-component monooxygenase system in Baeyer-Villiger oxidations, recombinant plasmids expressing Fred in tandem with the respective 2,5-DKCMO- and 3,6-DKCMO-encoding genes in *Escherichia coli* were constructed. Comparative substrate profiling of the isofunctional 2,5-DKCMOs did not yield obvious differences in Baeyer-Villiger biooxidations, but they are distinct from 3,6-DKCMO in the stereoselective oxygenations with various mono- and bicyclic ketone substrates.

In the history of *Pseudomonas* genetics, camphor (CAM) metabolism by *Pseudomonas putida* ATCC 17453 (NCIMB 10007; referred to here as strain PpCam), mediated by the “large” incompatibility group 2 (incP2) transmissible CAM plasmid, may be regarded as the oldest known profession of the genus (1–3). Pioneering work by the laboratory of the late Gunsalus and coworkers dates back half a century (1, 4–6). A schematic representation of the catabolic steps of conversion of the (+) and (−) isomeric forms of camphor (compounds 1 and 2, respectively) in strain PpCam that includes contributions from this study is shown in Fig. 1. In this pathway, the genetics and biochemistry of the cytochrome P450-containing enzyme complex (CamCAB) and 5-exo-hydroxycamphor dehydrogenase (CamD) that led to the formation of the 2,5-diketocamphane or 3,6-diketocamphane (compounds 3 and 4, respectively, in Fig. 1) are well understood (7–12).

Metabolism of compound 3 or 4 proceeds via the action of a Baeyer-Villiger monooxygenase (BVMO) system known as 2,5-diketocamphane monooxygenase (2,5-DKCMO) or 3,6-diketocamphane monooxygenase (3,6-DKCMO) (9, 13, 14). Both are prototype members of type 2 BVMOs (15), and they are flavin mononucleotide (FMN) and NADH dependent, in sharp contrast to the more frequently found type 1 BVMOs, which use flavin adenine dinucleotide (FAD) as a prosthetic group and NADPH as a cofactor. BVMOs in general are virtuous green reagents (using molecular oxygen as an oxidant and producing only water as a byproduct) that have a proven record of assessing high chemo-,

regio-, and enantioselectivity in a variety of oxidation reactions that include epoxidation and S- and N-heteroatom oxidations (16–20).

It is generally known that either 2,5-DKCMO or 3,6-DKCMO consists of a homodimeric FMN-containing oxygenation component and a second component that has been referred to as an NADH dehydrogenase or NADH oxidase (14) which may be common to both flavoproteins. In keeping with the modern nomenclature of the FMN-dependent two-component monooxygenase systems (21), we adopt the name flavin reductase, or Fred, in this study.

Both the DKCMO oxygenating subunits of strain PpCam have been purified to homogeneity and shown to be discrete enzymes

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This paper is dedicated to the memory of Irwin C. (Gunny) Gunsalus (1912 to 2008), whose middle initial, although it stands for Clyde, should be synonymous with camphor or cytochrome P450_{cam}.

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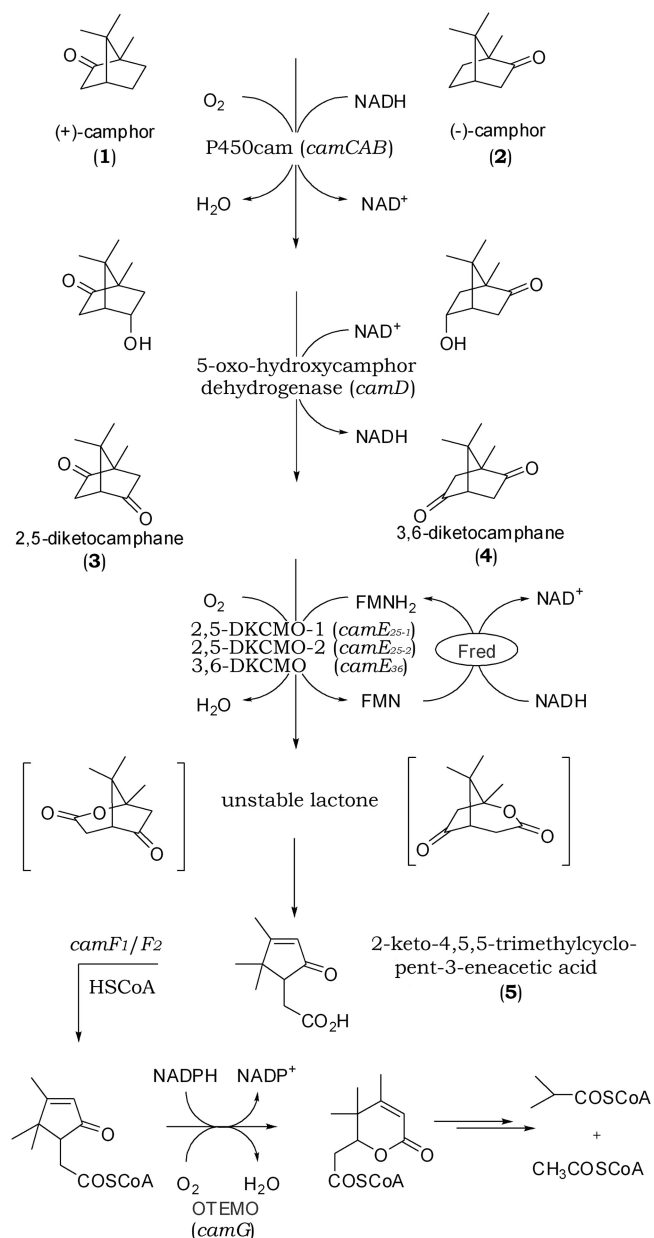


FIG 1 Metabolism of camphor isomers to acetyl-CoA and isobutyryl-CoA in *Pseudomonas putida* ATCC 17453. The figure was modified from Leisch et al. (29), with cumulative data from references 7, 9, and 11. Nomenclature of *camE* for the oxygenating components of diketocamphane (DKC) monooxygenase isozymes (DKCMO) was adapted from Koga et al. (10). Fred is a short-chain flavin reductase (this study). The lactones of DKC monooxygenations are presumed to undergo spontaneous hydrolysis to form compound 5 (9). *camF1* and *-F2* are putative genes for 2-oxo- Δ^3 -4,5,5-trimethylcyclopentenylacetyl-CoA synthetase (see Fig. S1 in the supplemental material) that produce the carbonyl-CoA (COSCoA) derivative of compound 5, a substrate for OTEMO (type 1 BVMO; 29). HSCoA, acetyl-CoA. Additional steps of the degradation pathway are discussed in Fig. S1 in the supplemental material.

having different molecular weights (subunit M_r , 37,000 to 40,000 versus 38,000 to 40,300) and isoelectric points (4.6 versus 5.5) (9, 13, 14). Also, both DKCMOs have been crystallized, but only the structure of 3,6-DKCMO has been solved to 2.00-Å resolution (Protein Data Bank identification no. [PDB ID] 2wgk) (22–24).

Recently, Kadow et al. (25, 26) described the structures of genes encoding a 2,5-DKCMO and 3,6-DKCMO and reported biotransformation results based on a presumed endogenous reductase from the *Escherichia coli* host that could complement the DKCMO activity. Needless to say, without a bona fide reductase, the reported activities were extremely low if the results are at all reliable. Indeed, the majority of the biotransformation experiments carried out in the past by the pioneering laboratory of Willetts using the two enantiomeric systems had been conducted in whole *P. putida* cells. A mixture of the 2,5-DKCMO and 3,6-DKCMO enzymes has been referred to as MO1 to distinguish it from the MO2 activity of 2-oxo- Δ^3 -4,5,5-trimethylcyclopentenylacetyl-coenzyme A (CoA) monooxygenase (OTEMO), a type 1 BVMO (15, 26–29). Importantly, each DKCMO enzyme was shown to have absolute specificity for substrates of the respective enantiomeric series of camphor ketones and was also shown to have useful enantioselective properties (27, 30–33). These enzymes are also active on the respective camphor enantiomers (13, 14, 34).

The following are brief accounts of previous attempts to isolate the reductase component of the DKCMO system. Conrad et al. (4–6) described this as an “electron transport oxidase” believed to catalyze the FMN-mediated reduction of oxygen to hydrogen peroxide by NADH. However, Trudgill et al. (35, 36) demonstrated that this enzyme of 36 kDa purified from (+)-camphor-grown cells of *P. putida* strain C₁B (ATCC 17453) (13, 14, 22) did not directly transfer electrons to oxygen and introduced the name NADH:(acceptor) oxidoreductase (also known by its trivial name, NADH dehydrogenase). The purified enzyme was found to bind FMN very weakly (dissociation constant [K_d] of 0.45 μ M) in a 1:1 ratio. It was further characterized to contain two flavin-binding sites and would loosely interact with the oxygenating component to form an active complex. Since these studies, there has been no further work on the enzyme.

In this study, we set out to identify and clone the gene for a flavin reductase (Fred) from strain PpCam that is requisite for oxygenating activity. The new Fred-encoding gene was assembled with the corresponding DKCMO gene for the first time to facilitate Baeyer-Villiger oxidations in a recombinant format. In addition, we applied pulsed-field gel electrophoresis (PFGE) to uncover salient new features of the prototypical CAM plasmid. This study was also designed to sequence CAM plasmid DNA beyond the well-established but limited *cam* operon locus with the goal of localizing the positions of the 2,5- and 3,6-DKCMO-encoding genes with respect to both known and potential new genes that can be assigned to the complete CAM degradation pathway.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. putida* ATCC 17453 (strain PpCam) and *E. coli* strains were grown at 30°C and 37°C, respectively, and routinely cultured in Luria-Bertani (LB) broth or media as previously described (29). When necessary, the media were supplemented with ampicillin (Ap; 100 μ g/ml). Growth of strain PpCam on (+)-camphor or (–)-camphor or racemic camphor (0.3 to 0.5%) as the sole carbon source was carried out in mineral medium as originally described (13, 14).

CAM plasmid size determination. Megaplasmid detection was first performed using the in-well cell lysis technique (37) as described in the information accompanying Fig. S2 in the supplemental material.

To determine the molecular sizes of the large plasmids, their profiles were determined by using nuclease S1 treatment followed by pulsed-field gel electrophoresis (PFGE) as originally described by Barton et al. (38) with some modifications. Briefly, genomic DNAs of *Sphingomonas aro-*

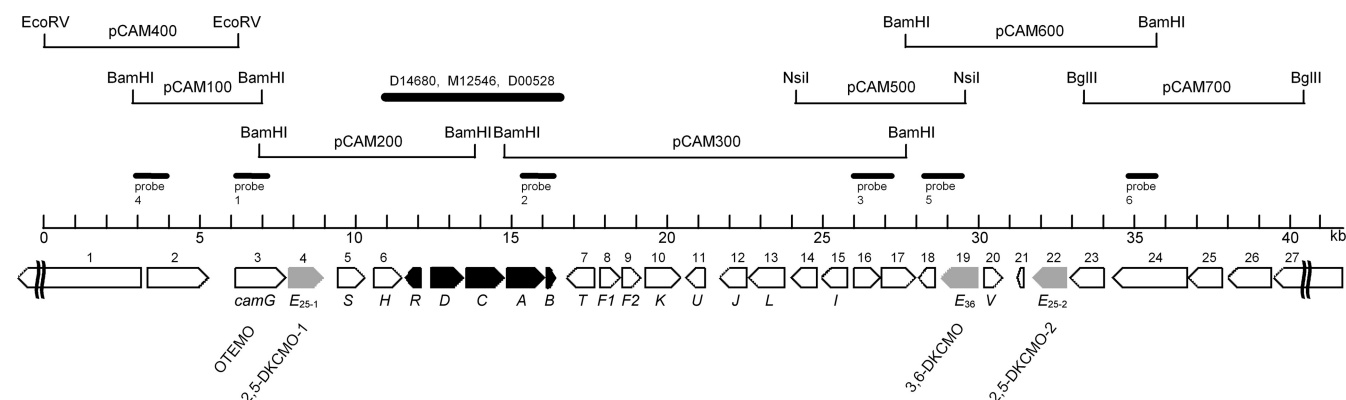


FIG 2 Localization of additional genes and predicted open reading frames (ORFs) flanking the established initial genes of the camphor *camDCAB* operon and its repressor, *camR*, on a ~40.5-kb sequenced region of the CAM plasmid of *P. putida* ATCC 17453. The predicted ORFs or genes are numbered from 1 to 27, except for the established *camRDCAB* genes, which are shaded in black. The orientation of the arrows indicates the direction of gene transcription. The candidate genes of this study (*camE₂₅₋₁*, *camE₂₅₋₂*, and *camE₃₆*) representing the three diketocamphane monooxygenase (DKCMO) isozymes are highlighted in gray. The previously established OTEMO-encoding gene (29) has been designated *camG* in accordance with the respective catabolic steps (see Fig. 1). *camS*, *-T*, *-U*, and *-V* are potential transcriptional regulators; *camV* is a close homolog of *camR*. Rationales for the predicted ORFs and the various subclones and the probe regions corresponding to coverage of the sequenced CAM plasmid locus are elaborated in the information accompanying Method SM1, Table S1, Table S2, and Fig. S1 in the supplemental material. The black solid line represents the previously sequenced region, with the indicated GenBank accession numbers.

maticivorans F199 used as a control (39) and PpCam were first embedded in agarose plugs. To linearize potential circular megaplasmids, 4-mm-thick slices of agarose plugs were cut out and equilibrated in S1 buffer (50 mM NaCl, 30 mM sodium acetate [pH 4.5], 5 mM ZnSO₄) for 30 min and then digested with 8 U of *Aspergillus oryzae* S1 nuclease (Fermentas EN0321) for 15 min at 37°C. Slices of the plug incubated only in S1 buffer were run in parallel to detect nonlinearized megaplasmids. The reaction was stopped by the addition of 20 µl of EDTA (0.5 M, pH 8). The plugs were loaded immediately on a 0.8% agarose gel in 1× Tris-borate-EDTA (TBE) buffer, and the wells were sealed by addition of agarose. The gel was run at 4°C for 65 h using a Q-Life Autobase PFGE system with ROM card no. 5 for resolution of 100- to 1,100-kb DNA fragments (40). The gel was stained with 0.5 µg/ml ethidium bromide for 1 h, washed in distilled water for 50 min, and then photographed.

Cloning and sequencing of additional genes in the CAM pathway. Recombinant techniques were carried out according to Sambrook et al. (41). Cloning and localization of the 2,5-DKCMO-1-encoding gene in a 6.8-kb BamHI fragment of PpCam total DNA in an *E. coli* recombinant plasmid (pCAM200) have been described previously (29). Additional cloning of an ~26-kb DNA segment of PpCam DNA downstream of the *camDCAB* operon in four overlapping clones (a 13-kb BamHI fragment in pCAM300; a 5.5-kb NsiI fragment in pCAM500; a 8.1-kb BamHI fragment in pCAM600; and a 7.1-kb BglII fragment in pCAM700) and analysis of the DNA sequence determination are described in the information accompanying Method SM1, Table S1, and Table S2 in the supplemental material. A summary of the established and predicted gene organization is shown in Fig. 2.

Construction of overexpression clones of DKCMOs. The isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *E. coli* pSD80 vector (42) was used to carry the respective *Pfu* DNA polymerase-amplified DKCMO-encoding genes using the forward and reverse primers with built-in EcoRI or PstI restriction sites listed in Table S1 in the supplemental material. The resulting clones transformed in *E. coli* BL21 cells were designated pDKCMO25-1, pDKCMO25-2, and pDKCMO36. The cloned inserts were verified by DNA sequencing as previously described (43, 44).

Purification of recombinantly produced DKCMOs. All purification procedures were performed at 4°C on an ÄKTAexplorer 100 air chromatography system (GE Healthcare). A crude enzyme extract of the corresponding stock culture was processed essentially as previously described for OTEMO (29). A three-step purification scheme that led to electropho-

retic purity of the proteins was developed as described in the information that accompanies Fig. S4 in the supplemental material.

CD spectroscopy and determination of T_m. Circular dichroism (CD) spectra of the DKCMOs were recorded on a Jasco J-815 spectrometer operating with Spectra Manager software. Temperature was controlled by a Jasco PFD-452S Peltier unit. Purified protein solutions were desalted using a HiPrep desalting column (26/10) previously equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The final protein concentration was adjusted to about 0.1 mg/ml, and the corresponding CD spectrum was recorded at between 200 and 260 nm using a Quartz cuvette (inner diameter = 0.1 cm). Blanks containing buffer only were prepared and used as a baseline. Temperature-dependent protein unfolding was monitored at 222 nm, with thermal profiles ranging from 20 to 80°C (2°C min⁻¹). Thermodynamic parameters (melting temperature [T_m], ΔH, ΔS, ΔG) were calculated using Spectra Manager software.

DKCMO cell-free assays. Enzyme activity was routinely detected in a reaction mixture (0.5 ml) containing Tris-HCl buffer (50 mM, pH 7.5), 3.3 mM NADH, 0.03 mM FMN, 30 mU of formate dehydrogenase (FDH), 50 mM sodium formate, 10 to 50 mU of Fred, and about 0.4 mg of the corresponding DKCMO. The reaction was started by adding (+)-camphor (2 mM) for the 2,5-DKCMOs and (–)-camphor for 3,6-DKCMO. Controls contained all components except DKCMO or Fred. Samples were incubated for 10, 20, 30, 60, 90 min, and the reaction was stopped by adding 0.5 ml of acetonitrile. Precipitated protein was removed by centrifugation, and substrate depletion was followed by high-performance liquid chromatography (HPLC) using a C₁₈ column. An isocratic method was used with H₂O/CH₃CN/CH₃COOH (598/400/2) as the mobile phase (0.5 ml/min) and UV detection at 220 nm on a Waters Millennium system.

Detection and purification of an FMN reductase (Fred) from strain PpCam. Fred activity was assayed in a reaction mixture (1 ml) containing Tris-HCl buffer (50 mM, pH 7.5), 0.07 mM NADH, and 0.025 mM FMN, and the reaction was initiated by the addition of an appropriate amount of enzyme. The decrease in absorbance at 340 nm due to the oxidation of NADH was monitored. Blanks containing all components except the substrate (FMN) were prepared. Specific activity was defined as the amount of protein that oxidizes one µmol of NADH (ε = 6.22 liters mmol⁻¹ cm⁻¹) per minute (U) per milligram of protein (U/mg).

Purification of Fred was performed at 4°C on an ÄKTAexplorer 100 air chromatography system (GE Healthcare). The crude extract of a (+)-

camphor-grown culture of PpCam obtained by cell breakage via the use of a French press was loaded on a DEAE-Sepharose FF column (XK50/20) equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The flow rate was 4 ml/min. The column was washed with the same buffer until no protein could be detected in the flowthrough, and the enzyme was subsequently eluted with a linear gradient of 0 to 0.2 M NaCl. Active fractions were pooled and concentrated by ultrafiltration (200 ml stirring cell; Amicon) using a YM3 membrane and applied to a nickel-nitrilotriacetic acid (Ni-NTA) column (16/10) previously equilibrated with 20 mM sodium phosphate buffer containing 0.15 M NaCl. The flowthrough containing the active protein was collected, concentrated, and applied to a HiLoad Superdex 200 prep grade column (16/60) which had previously been equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. Protein was eluted with the same buffer (flow rate of 1.5 ml/min) and collected in 2-ml fractions.

Kinetic parameters. Kinetic parameters of the Fred were determined by using double-reciprocal transformation (Lineweaver-Burk plot) of the Michaelis-Menten equation under steady-state conditions. Results were verified by Eisenthal-Cornish-Bowden direct plots. Initial reaction rates were measured at 25°C in Tris-HCl buffer (50 mM, pH 7.5) by using total substrate and a coenzyme concentration between 1 and 100 μ M, respectively.

N-terminal and internal peptide sequencing and chemical digestion of Fred. Purified Fred, separated by SDS-PAGE, was blotted to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). N-terminal sequence determination was performed with a sequencer (473A; Applied Biosystems) by the Edman method (45). Phenylthiohydantoin amino acids were analyzed by HPLC with a reversed-phase column. For internal peptide sequencing, purified Fred was adjusted to 1 mg/ml and chemically digested using the following chemicals: o-iodobenzoic acid (46), cyanogen bromide (47), and formic acid (48). Cleaved protein fragments were separated by SDS-PAGE (15% PA), and peptide sequences of the cleaved protein fragments were determined as described above.

Cloning of the flavin reductase-encoding gene from strain PpCam. Two primers with the following sequences were designed based on the determined peptide sequences (ATDPQWF and PPLVAF, respectively): fRED07-5'spec and 2SG-REV1 (see Table S1 in the supplemental material). Following the addition of a 3' overhang, the amplified 150-bp product was cloned in TOPO TA cloning vector pCR2.1-TOPO (Invitrogen catalog no. K4500-01) and transformed in *E. coli* Top 10, and the resulting plasmid was designated pCR2.1TOPO-frgPCR G1#3 and sequenced to confirm its identity. To clone the complete Fred-encoding gene, a GenomeWalker universal kit (Clontech catalog no. 638904) was utilized and genomic libraries from strain PpCam were generated by digestion with different blunt end-cutting endonucleases (EcoRV, PvuII, and StuI) and by adapter ligation at the ends of the resulting DNA fragments. These libraries were utilized as independent templates in three different PCRs. One gene-specific primer from the pCR2.1TOPO-frgPCR G1#3 sequence, GSP1RED (see Table S1 in the supplemental material), was used in combination with a kit adapter primer (AP1) in a first PCR. Subsequently, 1 μ l of the first PCR (diluted 50-fold) served as a template in a secondary PCR, applying one nested gene-specific primer, GSP2RED (see Table S1 in the supplemental material), along with a nested kit adapter primer (AP2). The resulting products were cloned into pCR2.1-TOPO, and the sequences of the insertions were determined.

The DNA fragment carrying the Fred-encoding gene was amplified by using Platinum Pfx DNA polymerase (Invitrogen) with two PCR primers with NdeI and EcoRI restriction sites (fred07Nde-f and fred07Eco-r in Table S1 in the supplemental material) to facilitate subsequent cloning. The amplified DNA fragment was purified from agarose gel, digested with the restriction enzymes, and cloned in the pET17b vector. *E. coli* BL21(DE3)/pLysS containing the plasmid pET-Fred07#3 was cultivated in 250 ml of LB medium containing 100 μ g/ml of ampicillin at 30°C. When the culture reached an optical density at 600 nm (OD_{600}) of 0.6, IPTG was added to reach a final concentration of 1 mM in the medium.

The cells were further cultured overnight and then harvested by centrifugation.

Construction of tandem clones in pSD80: CamE₂₅₋₁ plus Fred (CamE₂₅₋₁ + Fred), CamE₂₅₋₂ plus Fred (CamE₂₅₋₂ + Fred), and CamE₃₆ plus Fred (CamE₃₆ + Fred). All tandem clones were constructed using a MultiSite Gateway Pro kit (Invitrogen). For each DKCMO (CamE) clone, the *tac* promoter and the specific *camE* gene were amplified with Platinum Pfx DNA polymerase (Invitrogen) using the forward primer attB1MO (see Table S1 in the supplemental material) and reverse primers attB5rMO1, attB5rMO2, and attB5rMO3 (see Table S1 in the supplemental material) for *camE*₂₅₋₁, *camE*₂₅₋₂, and *camE*₃₆, respectively. The templates used for the amplification were clones pDCKMO25-1, pDCKMO25-2, and pDCKMO36. The T7 promoter and the Fred-encoding gene were amplified with Platinum Pfx DNA polymerase (Invitrogen) and primers attB5fred and attB2fred (see Table S1 in the supplemental material). The template for amplification was clone pET17b-Fred as described in the previous section.

The various *camE* PCR products were recombined with the pDONR 221 P1-P5r Gateway vector (Invitrogen) in separate reactions to form entry clones pDONR(2,5-DKCMO-1), pDONR(2,5-DKCMO-2), and pDONR(3,6-DKCMO). The PCR product of the Fred-encoding gene was recombined with the pDONR 221 P5-P2 vector to form entry clone pDONR(Fred07). pDONR(2,5-DKCMO-1) and pDONR(Fred07) were recombined with the pSD80 destination vector, which was constructed using the Gateway vector conversion system (Invitrogen) via ligation of the RfA cassette (provided in the kit) with vector pSD80, which had been linearized with the restriction enzyme BamHI and blunt ended with T4 DNA polymerase. The recombination product of the three plasmids was transformed into One Shot Mach1 T1 chemically competent *E. coli*. The plasmid containing the double clone (pSD80-CamE₂₅₋₁-Fred) was then transformed into *E. coli* BL21(DE3) for protein expression. Identical procedures were carried out for the recombination of pDONR(2,5-DKCMO-2) and pDONR(3,6DKCMO) with pDONR(Fred07) and the pSD80 destination vector to form plasmids pSD80-CamE₂₅₋₂-Fred and pSD80-CamE₃₆-Fred, respectively.

Monooxygenase-catalyzed BV oxidations. *E. coli* BL21 harboring the respective monooxygenases (2,5-DKCMO-1, 2,5-DKCMO-2, and 3,6-DKCMO) and a Fred gene-containing plasmid was maintained on LB medium containing glycerol (50% [vol/vol]) at -80°C. For biotransformation experiments, a fresh LB agar plate (1.5% agar) containing ampicillin (100 μ g/ml) was prepared from the stock culture, and one colony was transferred to a preculture (20 ml) containing LB medium supplemented with ampicillin (100 μ g/ml) and grown at 30°C at 200 rpm on an orbital shaker overnight. An aliquot of the suspension (2 ml) was used to inoculate LB medium (200 ml) supplemented with ampicillin (100 μ g/ml), and the resulting suspension was grown at 30°C at 200 rpm on an orbital shaker. At an OD_{600} of 0.5 (~3 h), protein expression was induced by the addition of IPTG (final concentration, 1 mM). The cells were allowed to grow for an additional 3 h (OD_{600} of 2.0 to 2.2), and the cell suspension was divided into 10-ml batches. A solution of substrate in isopropanol (1 M, 30 μ l) was added to each batch, and the reaction flask was shaken at 30°C at 200 rpm on an orbital shaker. After 18 h, the cell suspension was centrifuged and the supernatant was extracted with ethyl acetate (10 ml). The layers were separated, and the organic layer was dried over anhydrous sodium sulfate and filtered. The obtained solution was used for gas chromatography (GC) analysis. For retention times of starting materials and products and details on the GC analysis, readers are referred to Method SM2 in the supplemental material.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers AB771747 and KC349947.

RESULTS

Linear nature of CAM plasmid. The CAM plasmid extracted by in-well cell lysis was first analyzed by traditional agarose gel elec-

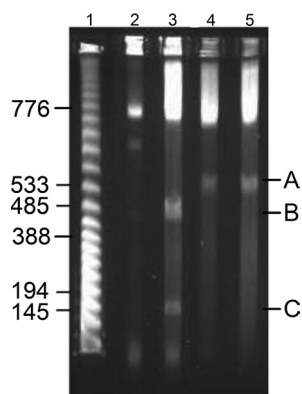


FIG 3 Establishing the linearity of the CAM plasmid of *P. putida* ATCC 17453 by pulsed-field gel electrophoresis. Lane 1, lambda ladder PFG marker DNA, with some sizes (in kb) indicated alongside; lanes 3 and 5, S1 nuclease-treated *S. aromaticivorans* and CAM plasmid DNA preparations, respectively. Lanes 2 and 4 correspond to non-S1 nuclease-treated samples of *S. aromaticivorans* F199 and CAM plasmid DNAs, respectively. A, B, and C indicate the estimated plasmid sizes of the CAM plasmid (533 kb) of strain PpCam, pNL2 (485 kb), and pNL1 (185 kb) of strain F199, respectively.

trophoresis and was seen to migrate as a discrete band, as were the two circular plasmids of *S. aromaticivorans* strain F199 strain (pNL1 and pNL2) that were used as controls (see Fig. S2 in the supplemental material). Next, S1 nuclease was used to linearize circular plasmids and analyzed on PFGE. As a result, two bands corresponding to the linearized pNL1 and pNL2 plasmids, calibrated by linear markers as being between 145.5 to 194 kb and 485 kb, respectively, are visible for the F199 strain treated with S1 nuclease (Fig. 3, lane 3). No such resolution was observed for the non-S1 nuclease-treated sample, as expected of large circular plasmids (lane 2). On the other hand, both the S1-treated (lane 5) and nontreated (lane 4) samples of CAM plasmid afforded one band estimated at 533 kb, indicating that the CAM plasmid is linear. Further, the migration of CAM plasmid exhibited constant mobility relative to the particular size marker under other pulse conditions (not shown).

Sequenced locus, gene context, and characteristics of 2,5-DKCMOs and 3,6-DKCMO. In total, a 40,450-bp region of the pCAM plasmid is now available as a result of cloning and sequencing various DNA fragments encompassing the established *camRDCAB* locus (Fig. 2). Except for the 2,5-DKCMO- and 3,6-DKCMO-encoding genes described below, characteristics of the other predicted open reading frames (ORFs) which may account for additional biochemical steps of the CAM pathway are given in Table S2 and Fig. S1 in the supplemental material.

As previously reported, a 2,5-DKCMO-encoding gene (now referred to as *camE*₂₅₋₁ for 2,5-DKCMO-1) was localized 88 bp downstream of the OTEMO-encoding gene (*camG*) and likely cotranscribed (29). Here we report the presence of a second copy of 2,5-DKCMO (*camE*₂₅₋₂ [for 2,5-DKCMO-2]) that is localized some 23 kb downstream and encoded on the opposite strand, implying divergent transcription (Fig. 2). In the same DNA strand, and separated by two potential ORFs downstream, the 3,6-DKCMO-encoding gene (*camE*₃₆) was identified. The 378-amino-acid protein sequence of 3,6-DKCMO was previously deposited in the RCSB protein data bank as code 2WGK (www.pdb.org), describing the dimeric structure of the protein (24).

Identification of the N-terminal portion of this protein was aided by the available 29-amino-acid peptide sequence (15, 31) with one mismatch (arginine to alanine change at position 20 [numbered from the first methionine]). It is noteworthy that the subunit structure of 3,6-DKCMO was superimposable with the α -subunit of luciferase, with a root mean square deviation (RMSD) of 1.83 Å (253 matching C α atoms of 378) (24).

The nucleotide sequences of *camE*₂₅₋₁ and *camE*₂₅₋₂ are 90% identical; the base changes result in 28 amino acid substitutions along the 363-amino-acid polypeptide, of which 18 are conservative changes (see Fig. S3a in the supplemental material). Interestingly, the predicted N-terminal 20-amino-acid sequence of 2,5-DKCMO-2 instead of 2,5-DKCMO-1 matches more closely to the peptide sequence obtained by N-terminal sequencing of what was then known as 2,5-DKCMO, a single protein (15, 31). The QA dipeptide at positions 2 and 3 is a key determinant (see Fig. S3b in the supplemental material).

Approximately half of the 2,5-DKCMO and 3,6-DKCMO polypeptide sequences are conserved (43.3% to 44.4% identity and 59.6% to 60.6% overall similarity), with the notable inclusion of two conserved stretches of 10 and 14 amino acids and a major deletion of 12 amino acids near the N termini of both 2,5-DKCMOs (see Fig. S3a in the supplemental material). A comparison of the predicted secondary structures between the 2,5-DKCMO isozymes and 3,6-DKCMO shows that sequence divergence appears to be localized in the extreme N-terminal region and around the deletion/insertion region.

During the process of isolation from the respective overproducing clones, all three proteins exhibited the characteristic intense yellow color of flavoproteins; however, the prosthetic group (FMN) appeared to be loosely bound to the proteins. When loaded on a hydrophobic column (butyl-S-Sepharose) to which the proteins bind, FMN was eluted as a clear yellow band upon washing. The now colorless enzymes, however, remained fully active in the *in vitro* assay where exogenous FMN was added. Some 25 mg/liter of purified 2,5-DKCMO-2 and 50 mg/liter of each of 2,5-DKCMO-1 and 3,6-DKCMO could be obtained from the respective overproducing clones. On SDS-PAGE, the purified 2,5-DKCMOs showed *M_r* values of 41,000 for either isozyme (theoretical values of 40,702 and 40,574, respectively) and 44,000 for the 3,6-DKCMO (theoretical value, 42,311) (see Fig. S4 in the supplemental material). That 2,5-DKCMO is not larger than 3,6-DKCMO agrees with the *M_r* estimated from the respective His-tagged proteins (25). Native *M_r* values analyzed on HiLoad Superdex 200pg were estimated as 60,000, 64,000, and 85,000, respectively (see Fig. S5 in the supplemental material), supporting the notion of the dimeric nature of the proteins as previously reported (13, 14, 22, 23).

All 3 DKCMOs exhibit the typical CD spectrum of proteins, with α -helices as the predominant form of secondary structure (see Fig. S6a in the supplemental material). They all show minima at 222 nm and 208 nm, with profiles that are nearly identical for the two 2,5-DKCMOs but somewhat different for the 3,6-DKCMO, especially at the 208-nm wavelength. Monitoring the CD at a fixed wavelength of 222 nm while adjusting the temperature allowed the visualization of the protein-unfolding process. Interestingly, the resulting estimated melting temperatures (*T_m*) of the proteins (the temperatures where the folded and unfolded proteins are in equilibrium) differed substantially. These are 56 $\pm$ 1°C, 63 $\pm$ 1°C, and 47 $\pm$ 1°C for 2,5-DKCMO-1, 2,5-DKCMO-2,

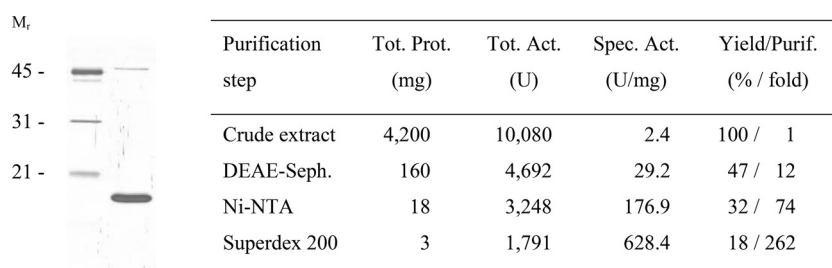


FIG 4 Purification scheme and SDS-PAGE (12% PA) analysis of 18-kDa FMN reductase (Fred) from *P. putida* ATCC 17453. Units of enzyme activity are defined by the amount of protein which oxidizes one μmol of NADH ($\epsilon = 6.22 \text{ liter mmol}^{-1} \text{ cm}^{-1}$) per min using FMN as the substrate. Protein standards (ovalbumin, carbonic anhydrase, and lysozyme) of decreasing sizes are indicated alongside. The purified Fred after the final size exclusion step is shown. Tot. Prot., total protein; Tot. Act., total activity; Spec. Act., specific activity; Purif., purification; Seph., Sepharose.

and 3,6-DKCMO, respectively (see Fig. S6b in the supplemental material). By virtue of its having a 7°C-higher T_m than that of 2,5-DKCMO-1 in the thermal denaturation experiments, it can be anticipated that 2,5-DKCMO-2 has a longer shelf life time than the other counterpart.

Identification, purification, and properties of Fred. In crude extracts of strain PpCam grown on (+)-camphor, FMN reductase activity (see Materials and Methods for details) was detected which seemed to originate from only one enzyme, which we designated Fred. This activity was not detected when the strain was grown with glucose as the sole carbon source. This enzyme was subsequently purified to homogeneity using a 3-step procedure with a yield of 18% (Fig. 4). The specific activity (628.4 U/mg) of the purified Fred represents an ~ 260 -fold purification. In the purification process, a major contaminating protein was experienced during the DEAE-Sepharose chromatography where medium components and about 90% of the unspecific proteins were removed. The protein that coeluted with Fred was subsequently identified by N-terminal sequencing as camphor-5 monooxygenase ($P450_{\text{cam}}$), which is known to be induced upon growth of strain PpCam on (+)-camphor. However, in the following Ni-NTA resin chromatography procedure, this major contaminant remained bound to the resin whereas the reductase flowed through and was collected and concentrated. The resulting homogenous protein fraction containing Fred was a clear solution showing a single absorbance maximum at 280 nm, indicating that it is a nonflavoprotein.

The active Fred was shown to be a homodimeric protein with an apparent M_r of 37,200 by size exclusion chromatography on Superdex 200 (see Fig. S7 in the supplemental material) and a subunit M_r of $\sim 18,000$ by SDS-PAGE (15% PA) analysis (Fig. 4). The pH optimum of 7.5 was determined in Tris/HCl buffer (50 mM). However, both the activity and pH optimum appeared to depend on the buffer used; e.g., at pH 7.5 in phosphate buffer, the enzyme activity was reduced to 40%; in piperazine-HCl (pH 5 to 6.5) and phosphate buffer (pH 6.5 to 7.5) (both at 50 mM), Fred was found to have optimum activity at a lower pH of 5 to 6 (not shown). The optimum temperature for Fred activity was seen at between 30 and 35°C.

Activation energy and thermostability. An activation energy value of 7 kcal mol^{-1} was estimated for the Fred reaction (see Fig. S8 in the supplemental material). Based on these data, a 10°C increase of the reaction temperature (ΔT_{10}) would result in a rate constant that is about 1.5 times higher. At 25°C, the free energy of activation ($\Delta G^\ddagger$) was calculated to be $11.7 \text{ kcal mol}^{-1}$.

Fred was rather stable when stored at 4°C over several days. However, at room temperature (25°C) the enzyme irreversibly unfolds with a half-life of about 80 min (see Fig. S9ab in the supplemental material). At higher temperature (between 30 and 35°C) the half-life of the enzyme is even shorter (~ 5 to 20 min).

Substrate specificity and kinetic properties of Fred. FMN and FAD are both substrates for the reductase. However, the enzyme favors FMN, as demonstrated by a 2-fold-higher rate constant and affinity that is about 5 times higher ($K_m = 3.6 \mu\text{M}$, $k_{\text{cat}} = 283 \text{ s}^{-1}$, $k_{\text{cat}}/K_m = 7.9 \times 10^7$) compared to that of FAD ($K_m = 19 \mu\text{M}$, $k_{\text{cat}} = 128 \text{ s}^{-1}$, $k_{\text{cat}}/K_m = 6.7 \times 10^6$). With regard to electron donors, only NADH is effective whereas NADPH at a similar concentration acts as a very poor cosubstrate. The K_m for NADH was estimated to be $32 \mu\text{M}$. NAD^+ is a competitive enzyme inhibitor with a determined K_i of 40 mM.

Cloning of the Fred-encoding gene and its sequence characteristics and gene context. Chemical cleavage of purified Fred using cyanogen bromide or formic acid yielded six peptide fragments (not shown), five of which were used to determine internal peptide sequences as well as N-terminal sequencing of the intact protein. The iodobenzoic acid-treated sample did not produce any usable fragment. Degenerate primers were designed from the sequenced peptides to first clone a part of the reductase gene and eventually the entire gene as described in Materials and Methods. In a sequenced 4.9-kb region, Fred is flanked by a potential GTP cyclohydrolase and luciferase upstream and three potential genes downstream, all being carried on the same DNA strand (see Fig. S10 in the supplemental material). Fred was cloned using the pET17b vector designated pET-Fred07#3 and overexpressed in *E. coli* BL 21(DE3)/pLysS.

Fred consists of 170 amino acids with a predicted M_r of 18,466, in good agreement with the experimentally determined result (Fig. 4). Three peptide sequences corresponding to amino acid positions 2 to 21, 8 to 29, and 45 to 68 helped to establish the identity of the protein (Fig. 5). A characteristic flavin reductase GDH motif (49) is found at positions 136 to 138. A conserved YGG motif (50) is found 5 to 7 residues away from the C terminus. In the BLAST search, the closest homolog (59% identity) is that of a "flavin reductase-like FMN binding protein" present in the genome sequence of *N. aromaticivorans* DSM 12444 (GenBank ABD25905.1). The closest homolog whose structure has been determined is nitrilotriacetate monooxygenase component B (NTA-MoB) (189 amino acids) derived from *Mycobacterium thermoresistibile* that was characterized as a homodimer with a split-barrel

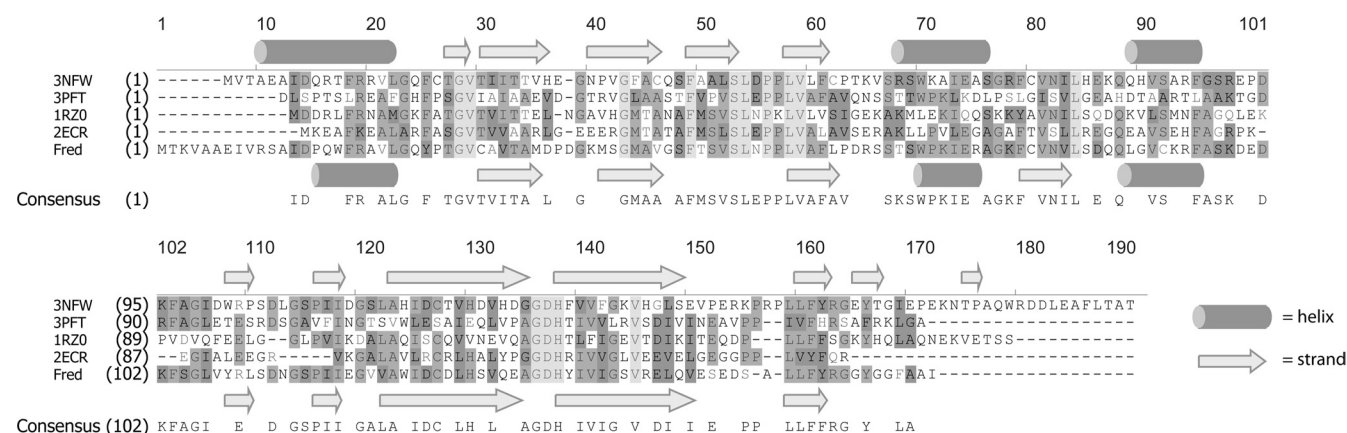


FIG 5 Protein sequence alignment of PpCam flavin reductase (Fred) with short-chain flavin reductases of known structures with top scores from the PDB entries. 3NFW, nitrilotriacetate monooxygenase component B of *Mycobacterium thermoresistibile* (40.5% identity); 3PFT, flavin reductase (DszD) of *M. goodii* (29.8% identity); 1RZ0, flavin reductase (PheA2) of phenol 2-hydroxylase of *Bacillus thermoglucosidasius* A7 (28.8% identity); 2ECR, flavin reductase (HpaC) of hydroxyphenylacetate 3-monooxygenase of *Thermus thermophilus* HP8 (25.1% identity). The secondary structural elements of 3NFW are indicated and labeled above the aligned sequences. The predicted secondary structural elements of Fred determined using the Psipred program (<http://bioinf.cs.ucl.ac.uk/psipred/>) are shown below. The consensus amino acids of the aligned sequences, including 10 entirely conserved residues, are highlighted.

motif typical of short-chain flavin reductases (PDB ID 3NFW) (51).

It is interesting that in a phylogenetic analysis, the PpCam Fred does not cluster with predicted flavin reductase counterparts originating from various *Pseudomonas* spp., e.g., *P. putida* F1 or strain KT2440, but rather clusters with members of the alphaproteobacteria, particularly members of the *Novosphingobium* and *Sphingomonas* genera (see Fig. S11 in the supplemental material).

The PpCam Fred-encoding gene is not part of the 40-kb sequenced *cam* locus or elsewhere, as shown by PCR amplification of the isolated CAM plasmid DNA (not shown). The same 4-kb gene locus is not found in any of the *P. putida* genomes sequenced so far (not shown).

Coexpression of DKCMO and Fred and enzyme activities. Evidence for protein coproduction of DKCMO tandemly expressed with Fred is shown in Fig. S12 in the supplemental material. Of the three enzyme pairs, 2,5-DKCMO-1 and Fred gave the clearest expression, as seen on the SDS-PAGE. For an unknown reason, the expression of 3,6-DKCMO appeared to be the weakest. Nonetheless, the biotransformation experiments (described in a later section) using whole cells support the notion of the coexpression of the two proteins. In contrast, whole cells expressing these oxygenating components alone showed very little activity with (+)- or (-)-camphor as the substrate.

In cell-free assays that involved an NADH-regenerating system using formate dehydrogenase (FDH) and sodium formate, addition of different amounts of Fred was seen to potentiate the monooxygenase activity. As an example, an ~20-fold increase in 2,5-DKCMO-2 activity due to the addition of Fred is shown in Fig. 6. In two other experiments, linear dependency on the concentration of Fred in the oxidation of (+)- or (-)-camphor was shown (see Fig. S13 in the supplemental material). These experiments also showed the high specificity of the respective DKCMOs for the camphor enantiomers. Moreover, a ratio of 4:1 (DKCMO:Fred) was found to be optimal for measuring enzyme activity. The pH optima for 2,5-DKCMO-1, 2,5-DKCMO-2, and 3,6-DKCMO were estimated to be 7.5, 8.0 (Tris-HCl buffer), and 7.0 (sodium phosphate buffer), respectively (not shown). Under these condi-

tions, the activities of the purified 2,5-DKCMO-1 and 2,5-DKCMO-2 *in vitro* were determined to be nearly identical at 1.0 U/mg and 1.1 U/mg for (+)-camphor. For 3,6-DKCMO, the activity was 0.81 U/mg with (-)-camphor.

Coupled DKCMO-Fred oxidations of selected ketones. To establish the substrate acceptance and enantioselectivity of the three DKCMOs, whole-cell oxidations were carried out for 18 h in shake flasks using 3 mM concentrations of various ketones. Reaction conversions and enantiopurities of starting materials and products were determined by chiral-phase GC as in the information accompanying Method SM3 in the supplemental material.

(i) Terpenones. For (+)-camphor (compound 1), 2,5-DKCMO-1-Fred and 2,5-DKCMO-2-Fred showed full conversion under the reaction conditions and did not convert (-)-camphor (compound 2) (Table 1). Conversely, 3,6-DKCMO-Fred fully converted (-)-camphor to lactone (compound 2a) and

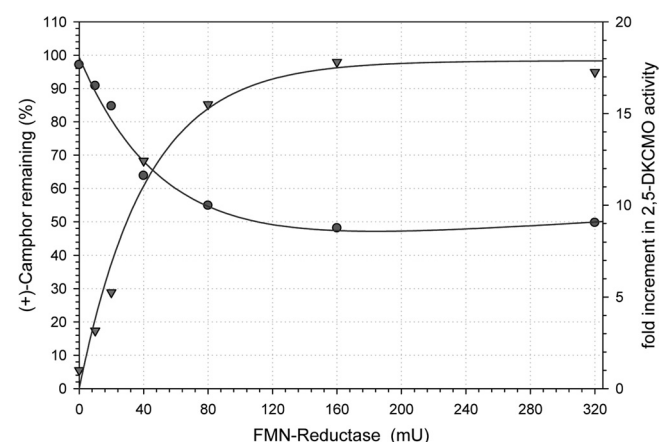


FIG 6 Potentiation of 2,5-DKCMO activity by the addition of FMN reductase (Fred). Remaining (+)-camphor (●) was monitored by HPLC with a constant amount of 2,5-DKCMO-2 and increasing Fred concentrations. Reaction incubation time was 30 min. The resulting increment in DKCMO activity is as indicated (▼).

TABLE 1 Oxidation of terpenones

Substrate	% oxidation			Product
	2,5-DKCMO-1 + Fred	2,5-DKCMO-2 + Fred	3,6-DKCMO + Fred	
	100	100	0	
	0	0	100	
	0	0	100	
	No conversion			
	No conversion			
	No conversion			

showed no conversion of (+)-camphor (compound 1). Similarly, 2,5-DKCMO and 3,6-DKCMO purified from camphor-grown PpCam were previously reported to be specific for (+)- and (-)-camphor, respectively (13). All three double clones were unable to convert (+)-fenchone (compound 6), (-)-fenchone (compound 7), and (+)-nopinone (compound 8).

(ii) **2-Substituted monocyclic ketones.** For the kinetic resolution of 2-substituted cyclohexanones (compounds 9 to 12), 2,5-DKCMO-1-Fred afforded the highest conversions and enantioselectivities among the three double clones (Table 2). 2,5-DKCMO-1-Fred oxidized the (*R*)-enantiomer of 2-phenylcyclohexanone (compound 12) with an $E > 200$. 2,5-DKCMO-2-Fred was also highly selective for the (*R*)-enantiomer ($E > 200$), although the

reaction proceeded to only 4% conversion compared to 23% for 2,5-DKCMO-1-Fred. In contrast, 3,6-DKCMO-Fred oxidized both enantiomers of 2-phenylcyclohexanone (compound 12) at the same rate ($E = 1$) and to only 2% conversion.

For the oxidation of alkyl-substituted cyclohexanones (compounds 9 to 11), all three clones were (*S*) selective, although 3,6-DKCMO-Fred recognized only 2-methylcyclohexanone (compound 9) ($E = 3.2$) as a substrate. 2,5-DKCMO-1-Fred and 2,5-DKCMO-2-Fred resolved 2-ethylcyclohexanone (10) with good enantioselectivities ($E = 43$ and 22, respectively) and oxidized 2-propylcyclohexanone (compound 11) with moderate enantioselectivities ($E = 19$ and 8.5, respectively).

For the kinetic resolution of 2-alkylcyclopentanones (compounds 13 and 14), 2,5-DKCMO-1-Fred resolved 2-*n*-hexylcyclopentanone (compound 14) with moderate enantioselectivity ($E = 19$) in favor of the (*S*)-enantiomer. 2,5-DKCMO-2-Fred oxidized 2-*n*-hexylcyclopentanone (compound 14) with low enantioselectivity ($E = 5$), whereas no conversion was observed with 3,6-DKCMO-Fred. All three clones were unable to convert 2-methylcyclopentanone (compound 13).

(iii) **4-Substituted cyclohexanones.** Biotransformations of 4-substituted cyclohexanones (compounds 15 to 18) by 2,5-DKCMO-1-Fred provided lactones with low to moderate ee values (Table 3). Oxidation of 4-methylcyclohexanone (compound 15) and 4-*n*-pentylcyclohexanone (compound 17) resulted in 27% and 26% ee for the respective (*R*)-lactones. Desymmetrization of 4-ethylcyclohexanone (compound 16) afforded the (*R*)-enantiomer at 71% ee, whereas the opposite enantiomer was obtained at 61% ee for the oxidation of *tert*-butylcyclohexanone (compound 18). 2,5-DKCMO-2-Fred also afforded the (*R*)-lactone of 4-ethylcyclohexanone (compound 16), although at a significantly higher (89%) ee. In contrast, the (*S*)-lactone was obtained at 87% ee with 3,6-DKCMO-Fred. 2,5-DKCMO-2-Fred also oxidized 4-methylcyclohexanone (compound 15) to the (*R*)-lactone (55% ee) but showed only traces of activity with the remaining ketones. Similarly, 4-methyl-, *n*-pentyl-, and *tert*-butylcyclohexanone were not substrates for 3,6-DKCMO-Fred.

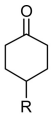
(iv) **Bicyclic ketones.** The bioconversion of [3.2.0]hept-2-en-6-one (compound 19) with 2,5-DKCMO-1-Fred yielded the “normal” as well as the “abnormal” lactone at a 1.3:1 ratio at 100%

TABLE 2 Kinetic resolution of racemic ketones^a

			2,5-DKCMO-1 + Fred		2,5-DKCMO-2 + Fred		3,6-DKCMO + Fred	
Substrate	<i>n</i>	<i>R</i>	Conv. %	<i>E</i>	Conv. %	<i>E</i>	Conv. %	<i>E</i>
9	1	Me	8	3.2 (<i>S</i>)	4	3.1 (<i>S</i>)	2	3.2 (<i>S</i>)
10	1	Et	11	43 (<i>S</i>)	6	22 (<i>S</i>)	0	n.a.
11	1	Pr	18	19 (<i>S</i>)	13	8.5 (<i>S</i>)	Traces	n.a.
12	1	Ph	23	>200 (<i>R</i>)	4	>200 (<i>R</i>)	2	1
13	0	Me	0	n.a.	0	n.a.	0	n.a.
14	0	He	6	12 (<i>S</i>)	3	5 (<i>S</i>)	0	n.a.

^a Percent conversion (Conv. %) values were determined by chiral GC based on area percentage. *E* values were calculated according to a program available at <http://biocatalysis.uni-graz.at/pub/enantio/>. Me, methyl; Et, ethyl; Pr, propyl; Ph, phenyl; He, *n*-hexyl; n.a., not available.

TABLE 3 Desymmetrization of prochiral ketones^a

						
15 16 17 18 R = methyl R = ethyl R = <i>n</i>-pentyl R = <i>tert</i>-butyl						
Substrate	2,5-DKCMO-1 + Fred		2,5-DKCMO-2 + Fred		3,6-DKCMO + Fred	
	Conv. %	ee %	Conv. %	ee %	Conv. %	ee %
15	8	27 (<i>R</i>)	5	55 (<i>R</i>)	Traces	n.a.
16	29	71 (<i>R</i>)	10	89 (<i>R</i>)	3	87 (<i>S</i>)
17	2	26 (<i>R</i>)	Traces	n.a.	0	n.a.
18	5	61 (<i>S</i>)	Traces	n.a.	0	n.a.

^a Percent conversion (Conv. %) values were determined by chiral GC based on area percentage. Percent ee (ee %) values were determined by chiral GC. n.a., not available.

conversion (Table 4). The “abnormal” lactone was obtained at an excellent ee (99%), whereas the ee of the “normal” lactone was significantly lower (77%). For 2,5-DKCMO-2-Fred, the “abnormal” lactone was obtained at an excellent ee (97%), and the ee of the “normal” lactone was also very high (87%). Purified 2,5-DKCMO from the native strain, which we assume is composed of both 2,5-DKCMO-1 and -2, was reported to give the “normal” and “abnormal” lactones in a 1.3:1 ratio, with product ee values of 82% and 100%, respectively (28). 3,6-DKCMO-Fred gave a 1:1.5 ratio of “normal” and “abnormal” lactones at 53% conversion. The “abnormal” lactone was obtained at 93% ee, and the “normal” lactone was obtained at 57% ee. Purified 3,6-DKCMO from the native strain (28) was reported to give a 1.3:1 ratio of “normal” and “abnormal” lactones at 30% reaction conversion. The ee values of the “normal” (10% ee) and “abnormal” (72% ee) lactones

were significantly lower than those obtained for the biotransformation with 3,6-DKCMO-Fred.

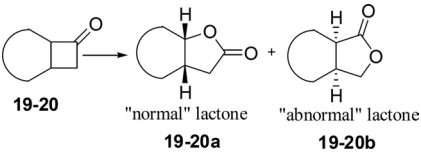
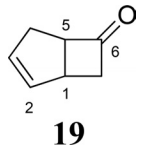
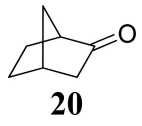
For the oxidation of norcamphor (compound 20) by 2,5-DKCMO-1-Fred, the “normal” lactone was formed in a 2.4:1 ratio with the “abnormal” lactone at ~60% reaction conversion. Product values were 40% ee and 57% ee, respectively. Biotransformation with 2,5-DKCMO-2-Fred yielded the “normal” lactone at 58% ee and the “abnormal” lactone at 20% ee and 59% reaction conversion. Purified 2,5-DKCMO was reported to give the “normal” lactone at 60% ee at 20% reaction conversion (28). Formation of the “abnormal” lactone was not reported. 3,6-DKCMO-Fred oxidized norcamphor exclusively to the “normal” lactone with excellent enantioselectivity (94% ee) at 26% reaction conversion. Similarly, purified 3,6-DKCMO was reported to give the “normal” lactone at >90% ee and 48% conversion (28).

DISCUSSION

We have purified a camphor-inducible flavin reductase (Fred) from strain PpCam, and this homodimeric protein of subunit *M_r* of 18,000 was shown to be functionally competent in providing the required electrons to the respective monooxygenase components in the oxidation of a variety of substrates. A chromosomally located Fred-encoding gene was cloned and enabled the first assembly of a bona fide two-component system in a recombinant format for the enantiomer-specific 2,5- and 3,6-DKCMOs. This DKCMO-Fred system represents the first among the known FMN-dependent two-component monooxygenase systems (21) where a single FMN reductase serves three separate monooxygenating components besides representing a single step in the CAM degradation pathway (Fig. 1).

In contrast to a previously described oxidoreductase (14, 35, 36), Fred is not a flavoprotein. The enzyme uses flavins (FMN in preference to FAD) only as the substrates, and thus it is not an NADH oxidase and is incapable of transferring electrons directly

TABLE 4 Regiodivergent oxidations of bicyclic ketones

				
Substrate	Biocatalyst	Conv. %	“Normal” lactone	“Abnormal” lactone
 19	2,5-DKCMO-1 + Fred	100	Ratio, 1.3:1	99% ee (1 <i>S</i> ,5 <i>R</i>)
	2,5-DKCMO-2 + Fred	100	77% ee (1 <i>R</i> ,5 <i>S</i>)	97% ee (1 <i>S</i> ,5 <i>R</i>)
	3,6-DKCMO + Fred	53	Ratio, 1.1:1	87% ee (1 <i>R</i> ,5 <i>S</i>)
			57% ee (1 <i>R</i> ,5 <i>S</i>)	93% ee (1 <i>S</i> ,5 <i>R</i>)
 20	2,5-DKCMO-1 + Fred	64 ^a	Ratio, 2.4:1	57% ee (1 <i>R</i> ,5 <i>S</i>)
	2,5-DKCMO-2 + Fred	59 ^b	Ratio, 1.8:1	20% ee (1 <i>R</i> ,5 <i>S</i>)
	3,6-DKCMO + Fred	26 ^c	58% ee (1 <i>R</i> ,5 <i>S</i>)	Normal lactone only
			94% ee (1 <i>R</i> ,5 <i>S</i>)	0

^a Starting material, 98% ee.

^b Starting material, 89% ee.

^c Starting material, 45% ee.

from NADH to molecular oxygen. Fred also differs from the purified NADH oxidase (14, 35, 36) in that it is not a single polypeptide of M_r 36,000 but consists of two identical subunits of M_r 18,000, making it highly unlikely that the homodimeric subunits would discriminate between FMN and FAD with regard to their binding capacities: 1 mol/mol FMN and 2 mol/mol FAD as described by Trudgill et al. (35). Other differences are in the isoelectric points (pI of 6.6 based on cellulose acetate electrophoresis for NADH oxidase versus a theoretical pI of 4.97 for Fred) and the amino acid compositions (1 methionine and 4 half-cysteines per mole of the former enzyme versus the sequence-derived 4 cysteines per subunit [8 in total] and 4 methionines per subunit [8 in total] for Fred).

Characteristics of the strain PpCam Fred classify it a new member of the class II nonflavoprotein reductases, meaning that the flavin acts as a substrate for catalysis in contrast to acting as a tightly bound cofactor in class I flavoprotein reductases (21, 52). The kinetic properties of Fred for FMN and NADH approximate those of FRD_H of *Beneckeia harveyi* (52, 53). The latter and a few others have been characterized to undergo a sequential mechanism of electron transfer; i.e., the reduced flavin substrate is transferred to the monooxygenase component only after its reduction by NADH (21). The conserved GDH motif in Fred implies that H136 may play a critical role in NADH binding and reduction of the FMN cofactor, as first reported for the NADH:flavin oxidoreductase (FRD_{Aa}) system of *Aminobacter animonvorans* (formerly *NmoB* or *NtaB* of *Chelatobacter heintzii*) (49). PpCam Fred also contains the conserved YGG motif reported in the modeled structure of a flavin reductase of *Thermus thermophilus* HB8 that uses FAD as the substrate (50). This region was deemed important in substrate flavin binding by deletion of the C-terminal 5 amino acids containing the YGG motif (50).

A second revelation of this study is the identification and localization on the CAM plasmid of a duplicated set of genes, *camE*₂₅₋₁ and *camE*₂₅₋₂, that encode 2,5-DCKMO-1 and 2,5-DCKMO-2. This unravels a 26-to-43-year-old mystery first documented by Gunsalus and coworker (54) and later by Taylor and Trudgill (14), who, although they could not reproduce the presence of a “third” component, referred to as *E*₂′ in reference 54, provided electrophoretic evidence of two separable forms of 2,5-DCKMO. The predicted charge difference between the two isozymes (pI 5.58 for DCKMO-1 and 5.40 for DCKMO-2) would account for the electrophoretic separation in a native gel (14). Clearly, the high sequence identity between *camE*₂₅₋₁ and *camE*₂₅₋₂ was the result of a gene duplication event (and of sequence divergence in the case of *camE*₃₆). Identification of the duplicated set of 2,5-DCKMO-encoding genes means that Kadow et al. (25) had cloned the *camE*₂₅₋₁ and that all previously reported biotransformation results obtained using whole cells of PpCam most likely represented the summation of the two DCKMO activities. Interestingly, the N-terminal amino acid sequence experimentally determined for one DCKMO (15, 31) actually has a better match to that of DCKMO-2 than DCKMO-1 (see Fig. S3b in the supplemental material).

With the reconstituted and “personalized” DCKMO-Fred plasmid systems, we confirmed the enantiomeric specificity of the 2,5-DCKMO and 3,6-DCKMO enzymes for the (+) and (−) camphor as previously reported (13, 14). On the other hand, contradictory results were presented by Kadow et al. (25, 26), who relied on an unknown factor in *E. coli* to effect substrate oxida-

tions. Given the current spectrum of substrates, the oxidations seen with the two 2,5-DCKMO isozymes so far appear indistinguishable. For synthetic purposes, the most notable result is the specificity toward the (*R*)-enantiomer of 2-phenylcyclohexanone (compound 12), which rendered an *E* value of >200 in both cases (Table 3). The action of 3,6-DCKMO is most different with regard to oxidation of norcamphor (compound 20), where it provided the “normal” lactone exclusively, with excellent enantioselectivity (94% ee) at 26% reaction conversion (Table 4).

Although the CAM plasmid is widely known as one of the oldest known degradative plasmids, alongside the SAL, NAH, and OCT plasmids that are responsible for the degradation of salicylate, naphthalene, and octane, respectively (2, 55), its molecular size has been largely propagated in the literature as 150 MDa or 236 to 250 kb (see, e.g., reference 25). Here, by the criteria of PFGE, the CAM plasmid was determined to have a size of ~530 kb, besides being linear. A covalently closed circular plasmid would exhibit a constant migration pattern in the PFGE that is independent of the pulse conditions (see the Method SM2 note in the supplemental material).

Per the gene elucidation of the CAM degradation pathway, eight catabolic genes responsible for four biochemical steps besides the regulatory *camR* are now presently known and characterized (8, 10–12, 24, 25, 29) (this study). By sequence homology, additional ORFs that could account for the remaining steps of the degradation pathway are presented in Fig. S3 in the supplemental material. Note that besides the well-characterized TetR-type *camR* repressor that regulates the P-450cam hydroxylase operon (56), four potential transcriptional repressors (orf5[CamS], orf11[CamU], and orf20[CamV] of the TetR type and orf7[CamT] of the LysR type) with limited sequence identity to each other, with the exception of Orf20[CamV] and CamR (54% identity), decorate the sequenced *cam* locus (Fig. 2). Clearly, regulation of the entire *cam* pathway is complicated and is beyond the scope of this study. Suffice it to say that the *camE*₂₅₋₁ and *camG* genes and the *camE*₂₅₋₂ and *camE*₃₆ genes are predicted to be divergently transcribed per the position on the opposite DNA strand. Interestingly, this supports an earlier observation that the MO1 (mixtures of 2,5- and 3,6-DCKMO) and MO2 (OTEMO) activities are not “coordinately controlled” (27).

In conclusion, the availability of the recombinant format of the three DCKMO-Fred systems opens up new opportunities in organic synthesis besides the production of camphor lactones. Access to exquisite sulfoxide synthons by “separated” DCKMO is a potentially grand opportunity previously made possible by using “washed whole cells” of strain PpCam (30). With regard to mechanistic study, the new DCKMO-Fred systems provide a unique model for structure-function analyses of a special enantiocomplementary kind where a single flavin reductase serves three separate monooxygenating components. Whether there are differences in the mechanism of flavin transfer from the reductase to the DCKMO enantiomeric pair is just one more issue with respect to the complexity of flavoenzymology (21, 57).

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