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Heterologous expression and characterization of the ene-reductases from *Deinococcus radiodurans* and *Ralstonia metallidurans*

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ABSTRACT

The Old Yellow Enzyme (OYE) homologues or ene-reductases (ER) from *Deinococcus radiodurans* (*Dr*ER) and *Ralstonia metallidurans* (*Rm*ER) were cloned and characterized. Sequence and phylogenetic analysis revealed both these enzymes to belong to the YqjM-like or "thermophilic-like" group of OYEs, both sharing more than 60% sequence similarity to the ER from *Thermus scotoductus*. This group of OYEs is characterized by a conserved cysteine residue modulating the redox potential of the flavin cofactor as well as a conserved tyrosine residue located at the N-terminus region involved in binding certain ligands. The genes were recombinantly expressed in *Escherichia coli* as functional soluble proteins. Both ERs have monomer molecular weights of approximately 40 kDa, with *Dr*ER a homodimer in solution and *Rm*ER a monomer. *Dr*ER and *Rm*ER are optimally active at pH 7–7.5 at 30 °C and 35 °C respectively. Although the enzymes showed comparable affinities towards the ubiquitous ER substrate 2-cyclohexenone, the specific activity and catalytic efficiency of *Dr*ER were more than twice those observed for *Rm*ER. Similar to other members of this subclass of ERs, no conversion was detected with cyclic Cβ substituted enones, and only *Dr*ER was able to convert citral. Both *Dr*ER and *Rm*ER were highly active at reducing N-phenyl substituted maleimides. The selectivity of the ERs was assessed using both the isomers of carvone, which were converted with high diastereomeric excesses. Ketoisophorone and 2-methylcyclopentenone were converted to their (*R*)- and (*S*)-enantiomeric products respectively. Finally, a light-driven cofactor regeneration system was used to drive enzymatic reduction in the absence of NAD(P)H.

1. Introduction

The Old Yellow Enzyme (OYE) family or ene-reductases (ER) is a ubiquitous group of flavoproteins that catalyze the asymmetric reduction of activated C=C bonds of a wide variety of α/β-unsaturated carbonyl compounds. First described as a yellow enzyme involved in the oxidation of NADPH by molecular oxygen, Old Yellow Enzyme was the first flavin-containing enzyme characterized and played an important role by serving as model enzyme in studies aimed at understanding the role of flavin cofactors in proteins [1,2]. Twenty years after Stott and co-workers [3] reported 2-cyclohexenone as an alternative electron acceptor for OYE1 from *Saccharomyces pastorianus* (formerly *carlsbergensis*), the substrate range of OYE has broadened to now include a variety of cyclic and acyclic enones and enals (for recent reviews see [4,5]).

The reduction of these activated alkenes occurs through a ping-pong bi–bi mechanism whereby both the oxidant and reductant bind in the same active site sequentially. NAD(P)H is oxidized by hydride transfer to the flavin mononucleotide (FMN) cofactor, followed by the subsequent binding of the substrate and the concerted transfer of the hydride from the reduced FMN to the Cβ of the alkene and a proton to the Cα [6]. This proton is donated from an active-site tyrosine, or can be directly derived from the solvent [7,8]. Importantly, the oxidative half-reaction results in a net *trans* addition which occurs with absolute stereospecificity. OYEs have therefore become attractive biocatalysts due to their ability to perform *trans*-hydrogenation [9] as an alternative to current chiral organometallic catalysts [10] for enantioselective hydrogenation. This asymmetric reduction yields enantiopure chiral building blocks, important in the synthesis of various fine chemicals and pharmaceuticals [11]. While the physiological function of these enzymes remains elusive, additional substrates and activities have been identified, including the reduction of α,β-unsaturated dicarboxylic acids and dimethyl esters [12], maleimides [13,14], nitroalkenes [15,16], steroids [17] as well as the reductive denitration of nitrate esters and nitroaromatics [18–20].

The list of OYEs has also been growing steadily to now include OYE homologous characterized, both biocatalytically and structurally, from yeast [21], plants [22,23], bacteria [24–27] and recently thermophilic bacteria [28–30], allowing biocatalysis at

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higher temperatures. Although the overall structure and mechanism of these OYE homologues are conserved, slight changes within the catalytic site architecture of these enzymes are yielding different stereo-specificities. These stereopreferences have recently been structurally investigated to predict biocatalytic properties based on sequence data [31]. In addition, directed evolution studies to broaden substrate scope as well as to engineer reversed stereochemical outcomes through different substrate binding orientations [32–34] are also elucidating critical factors in substrate specificity and selectivity, in addition to expanding our catalytic toolbox.

One of the subclasses of ERs, the so-called YqjM-like or "thermophilic-like" OYEs is dominated by the recently described ERs from thermophiles [5]. Only two mesophilic counterparts are found within this subclass, YqjM from *Bacillus subtilis* [26] and XenA from *Pseudomonas putida* [25]. Here we report the cloning, heterologous expression and characterization of two new mesophilic ene-reductases from *Deinococcus radiodurans* and *Ralstonia metallidurans*, belonging to this underrepresented subclass. Finally, we investigate the use of a light-driven co-factor regeneration system with both ERs for the photobiocatalytic reduction of C=C bonds.

2. Experimental

2.1. Bacterial strains and culture conditions

Deinococcus radiodurans type strain and *Ralstonia* (*Cupriavidus*) *metallidurans* strain CH34 was obtained from the Leibniz Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). *D. radiodurans* was cultured in nutrient broth consisting of 5 g/L peptone and 3 g/L meat extract (pH 7, 200 rpm, 30 °C) and *R. metallidurans* was cultured in *Corynebacterium* broth consisting of 10 g/L peptone, 5 g/L yeast extract, 5 g/L glucose and 5 g/L NaCl (pH 7.2, 200 rpm, 35 °C). *E. coli* strains were grown in Luria–Bertani (LB) medium at 37 °C with shaking (200 rpm).

2.2. Cloning and heterologous expression of the ERs

Genomic DNA (gDNA) was isolated from the *Deinococcus* and *Ralstonia* strains using Aquapure Genomic DNA kit (Bio-Rad) as per manufacturer's instructions. The complete open reading frames (ORFs) were PCR amplified using the Expand high-fidelity PCR system (Roche) with oligonucleotides DR_F1_Nde (5′ CAT ATG ACC GTG TCT TCC GCC GCT GCA CC 3′) and DR_R1_Eco (5′ GAA TTC TTA CCA CCC CGC CCG CGC GTA CTG 3′) for the ER from *Deinococcus* and RM_F1_Nde (5′ CAT ATG CCT CAT CTC TTC GAT CCG TAC C 3′) and RM_R1_Eco (5′ GAA TTC TCA ACG CTG GCC GAA GTG CGC GT 3′) for the ER from *Ralstonia*. Primers contained *Nde*I and *Eco*RI restriction sites (underlined) for directional cloning and incorporation of a N-terminal hexahistidine-tag and thrombin cleavage site from the pET vector. Reaction mixtures (50 μL) consisted of 10× Expand high-fidelity buffer with 15 mM $MgCl_2$ (5 μL), deoxynucleoside triphosphates (0.2 mM each), Expand high-fidelity enzyme mix (3.5 U), 50 ng of gDNA, and 0.2 μM of both the forward and reverse primers. PCR conditions consisted of an initial denaturing step at 95 °C for 5 min, followed by 25 cycles of denaturing at 95 °C (30 s), annealing at 62 °C (40 s), and elongation at 72 °C (1.5 min), with a final extension at 72 °C for 10 min. Purified PCR products (Biospin gel extraction kit, BioFlux) were ligated into pGEM-T Easy (Promega) and transformed into *E. coli* TOP10 competent cells (Invitrogen) and selected for on LB-plates containing 100 μg/mL ampicillin. Plasmid DNA was isolated (Biospin plasmid DNA extraction kit, BioFlux) and verified by DNA sequencing. For expression of the ene-reductases, the ORFs were sub-cloned into pET28b(+) (Novagen) using the *Nde*I and *Eco*RI restriction sites. The pET28-ene-reductase constructs were transformed into *E. coli* BL21(DE3) competent cells (Lucigen) and selected on LB-plates containing 30 μg/mL kanamycin.

2.3. Heterologous expression and protein purification

Expression of the ene-reductases was performed using auto-induction media (ZYP5052 medium; [35]) at 25 °C (200 rpm) for 24 h. Cells were harvested through centrifugation (5000×*g*, 15 min) and washed twice and resuspended (1 g wet weight cells in 10 mL buffer) in 20 mM MOPS-NaOH (pH 7.4) and 0.1 M NaCl buffer. Cells were broken using a One Shot Cell Disruption system (Constant Systems Ltd) at 30 KPSI. Unbroken cells and debris were removed through centrifugation (5000×*g*, 20 min). The soluble fraction (cytoplasm) was obtained through ultracentrifugation (100,000×*g* for 90 min).

Recombinant N-terminally His_6-tagged proteins were purified by immobilized metal-affinity chromatography (IMAC) and size-exclusion chromatography. The soluble fractions were loaded onto HisTrap FF columns (5 mL, GE Healthcare) equilibrated with 20 mM MOPS-NaOH (pH 7.4) containing imidazole (40 mM) and NaCl (0.5 M). Unbound proteins were removed by washing (5 mL/min) with the same buffer. Recombinant ERs were then eluted in the same buffer with use of an increasing linear gradient (100 mL) of imidazole up to 0.5 M. Fractions containing the characteristic yellow colour were pooled for subsequent purification. All protein samples were incubated with excess FMN before size-exclusion chromatography (SEC). Samples were concentrated to approximately 3 mL by ultrafiltration (30 kDa MWCO, Millipore) and loaded onto a Sephacryl S-200HR columns (65 × 2.5 cm, Sigma) equilibrated with 20 mM MOPS-NaOH (pH 7.4) and 0.1 M NaCl. Proteins were eluted with the same buffer at a flow speed of 1 mL/min. Alternatively, samples from the Ni-affinity chromatography step were desalted using PD-10 desalting columns (GE Healthcare) into 20 mM MOPS-NaOH and 0.1 M NaCl (pH 7.4). Protein concentrations were determined with the BCA protein assay kit (Pierce) with bovine serum albumin (BSA) as standard. Enzyme purity was evaluated using SDS-PAGE [36] stained with Coomassie brilliant blue R-250. PageRuler protein ladder (Fermentas) was used as molecular mass markers. Quaternary structures of the ERs were determined by SEC using Gel Filtration standards (Bio-Rad) and BSA.

2.4. Enzyme assays

Steady-state kinetics of the purified ene-reductases were performed by measuring the rate of NAD(P)H oxidation at 340 nm (Cary 300Bio UV/Vis spectrophotometer) with use of an extinction coefficient of 6.22/mM/cm. Assays were performed in 1 mL reaction volumes containing NADPH (0.3 mM), 2-cyclohexen-1-one and the purified protein [*Rm*ER = 10–13 μg (0.24–0.31 nmol); *Dr*ER = 5–6 μg (0.12–0.15 nmol)]. Reactions were performed in 20 mM MOPS-NaOH (pH 7.4) containing 0.1 M NaCl at 30 °C. Assays were performed under aerobic conditions and NADPH oxidation by the enzyme due to molecular oxygen was measured independently and subtracted from the total oxidation rates with substrates.

Biotransformation for substrate scope and selectivity analysis were performed in 1 mL reaction volumes consisting of 2 mM NADH and 1 mM substrate, purified enzyme (20 μg) in 20 mM MOPS-NaOH (pH 7.4) with 0.1 M NaCl buffer at 30 °C. Conversions were determined after 5 h of incubation. For GC–MS analysis, reaction mixtures were extracted using an equal volume of ethyl acetate and samples separated on a FactorFour VF-5ms column (30 m × 0.25 mm × 0.25 μm, Varian). Chiral separation of the reduction products of *R*- and *S*-carvone, 2-methylcyclopentenone and ketoisophorone were performed on a Astec Chiraldex G-TA column (30 m × 0.25 mm × 0.25 μm, Sigma–Aldrich) and compared to