Journal of Molecular Catalysis B: Enzymatic 99 (2014) 89–95

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

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

S. Litthauer[a], S. Gargiulo[b], E. van Heerden[a], F. Hollmann[b], D.J. Opperman[a,*]

[a] University of the Free State, Department of Biotechnology, 205 Nelson Mandela Drive, Bloemfontein 9300, South Africa
[b] Delft University of Technology, Department of Biotechnology, Julianalaan 136, 2628BL Delft, The Netherlands

ARTICLE INFO

Article history:
Received 20 August 2013
Received in revised form 3 October 2013
Accepted 25 October 2013
Available online 1 November 2013

Keywords:
Old Yellow Enzyme
Ene-reductase
α,β-Unsaturated carbonyl
Cofactor regeneration
Flavin oxidoreductase

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

* Corresponding author. Tel.: +27 51 401 2714; fax: +27 51 401 9376.
E-mail address: opperdj@ufs.ac.za (D.J. Opperman).

1381-1177/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molcatb.2013.10.020

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

reference activities [37] or the MS fragmentation of diastereomers [38]. For HPLC analysis, reactions were stopped by the addition of 10 μL of concentrated HCl and samples separated on a Jupiter 5 μm C18 300 Å column (250 mm × 4.6 mm, Phenomenex).

2.5. Co-factor regeneration

The efficacy of a light-driven cofactor regeneration system in the reduction of 2-cyclohexenone using both *Dr*ER and *Rm*ER was investigated. Reactions were performed on a 10 mL scale in closed glass Hungate tubes and consisted of 5 mM EDTA and substrate, 0.3 mM FMN (0.05 mM for prochiral substrates) and 0.1 mg/mL (2.4 μM) enzyme in 20 mM MOPS-NaOH (pH 7.4) with 0.1 M NaCl buffer at 30 °C. Oxygen was removed from the reaction mixture by passing N_2 gas through the reaction mixture for 10 min before the enzyme was added. The reaction mixture was illuminated for 24 h with samples withdrawn under a N_2 gas stream and extracted with one volume equivalent of ethyl acetate for GC–MS analysis. Light was provided as cold light by a KL 1500 compact (Leica) fitted with a 150 W bulb (5 cm distance).

2.6. Phylogenetic analysis

Old Yellow Enzyme homologue sequences were retrieved from the National Centre for Biotechnology Information (NCBI). Multiple sequence alignments of the amino acid sequences were performed using the MUSCLE EBI web tool (http://www.ebi.ac.uk/Tools/msa/muscle) with the default parameters [39]. The best amino acid substitution model was estimated for tree building using the MEGA 5 software [40]. The Whelan and Goldman (WAG) model [41] was selected with a discrete Gamma distribution with 5 rate categories and by assuming that a certain fraction of sites are evolutionary invariable. An un-rooted maximum likelihood tree was constructed using MEGA 5. The phylogenetic tree was inferred using nearest-neighbour-interchange (NNI) with bootstrap support for individual nodes calculated on 500 replicates.

3. Results and discussion

3.1. Cloning of the ene-reductase

Similarity searches using BLASTp of whole-genome sequences identified two open reading frames (ORFs) encoding for typical orthologues of Old Yellow Enzyme/ene-reductases in the *D. radiodurans* genome (*Dr*ER and *Dr*ER2) and only one in *R. metallidurans* (*Rm*ER). The protein sequences of the *Dr*ERs share only 37% similarity with each other, with *Dr*ER2 also showing an unusually long C-terminus, more than 50 residues longer than *Dr*ER. The 1113 bp and 1116 bp ORFs encoding the putative ene-reductases, *Rm*ER and *Dr*ER, were successfully amplified from genomic DNA, whereas no PCR product could be obtained for *Dr*ER2. Both these ene-reductases showed the highest similarity at protein level to the ER characterized from *Thermus scotoductus* (*Ts*ER) [29,42] with 48% and 46% identity (61% and 60% similarity) for *Dr*ER and *Rm*ER respectively. Multiple alignments with previously cloned and characterized ene-reductases and phylogenetic analysis (Fig. 1) showed both these putative ERs to group with the YqjM-type (or "thermophilic-like") ERs that also include XenA from *Pseudomonas putida* [25] as well as the OYEs from the thermophiles *T. scotoductus*, *Thermoanaerobacter pseudethanolicus* (*T*OYE; [30]) and *Geobacillus kaustophilus* (*Gk*OYE; [28]).

This subclass of ERs all has a conserved histidine pair (His175 and His172, *Ts*ER numbering) for the hydrogen bonding of the substrate's carbonyl oxygen, as well as a conserved proton donating tyrosine for the oxidative half reaction (Tyr177). The YqjM-like group of ERs has a conserved cysteine (Cys25) residue, whereas all other "classical" OYEs have, similar to OYE1, a corresponding conserved threonine (Fig. 1). This cysteine has been implicated in modulating the reduction potential of the flavin [43]. This group also has a conserved Tyr (Tyr27) near the N-terminus which has been shown to participate in the binding of certain ligands [26,29]. The Arg-finger (Arg347) observed in *Ts*ER and YqjM appears to be conserved, with only XenA having a tryptophan residue from the neighbouring monomer extending into the catalytic site. The multiple alignments also revealed *Dr*ER and *Rm*ER to vary notably in the length of their N- and C-termini. *Dr*ER and *Rm*ER contain more than 12 additional residues on their N- and C-termini respectively, relative to each other as well as other members of this group.

3.2. Protein expression, purification and physicochemical characterization

Both *Dr*ER and *Rm*ER were expressed as soluble N-terminally histidine-tagged variants with apparent monomer molecular weights of approximately 38 kDa (Fig. 2). The ERs were purified to homogeneity with typical yields between 160 and 200 mg/L *E. coli* culture using IMAC and size-exclusion chromatography (SEC; Fig. 3). SEC also showed the *Dr*ER to exhibit a homodimeric quaternary structure similar to *Ts*ER, whereas *Rm*ER occurred as monomers in solution, possibly due to its extended C-terminus or possibly by the interference by the hexahistidine tag and thrombin-cleavage site from the pET vector [44]. Although sequence based analysis shows the Arg-finger to be conserved in *Rm*ER, its monomeric structure suggests a more open catalytic site without the Arg-finger from an adjacent monomer. The thermophilic-like ERs tend to vary greatly in their quaternary structure, with *Ts*ER occurring as dimers in solution [29] and *T*OYE from *Thermoanaerobacter* even occurring in multiple oligomeric states (tetramers to dodecamers) *in vitro* [30].

The purified proteins had an intense yellow colour due to the bound flavin (FMN) and physicochemical characterization revealed that both enzymes function optimally near the optimal growth temperature and pH of their original bacterial sources. The optimum pH and temperature conditions for both the *Dr*ER and *Rm*ER was determined to be 7–7.5 at 30 °C and 35 °C respectively.

3.3. Substrate scope and kinetic characterization

Both enzymes catalyzed the reduction of 2-cyclohexen-1-one through the oxidation of NADPH or NADH, with a preference for NADPH as electron donor (Fig. 4). Substrate inhibition was observed when NADH was used as electron donor. As ERs function through compulsory ordered substrate binding and product release through a ping-pong bi–bi mechanism, a lower binding affinity for NADH can cause 2-cyclohexenone to act as a competitive inhibitor at high concentrations for the reductive-half reaction, effectively binding before the enzyme is reduced (dead-end inhibition). Saturation kinetics with NADPH (Table 1) revealed similar affinity (K_m) of both enzymes for 2-cyclohexenone, although *Dr*ER had a higher specific activity compared to *Rm*ER, making the catalytic efficiency (k_{cat}/K_m) of *Dr*ER almost three times higher than that observed for *Rm*ER towards cyclohexenone.

To explore the substrate scope and selectivity, a range of potential substrates were investigated (Table 2). Biotransformation reactions were performed using purified enzymes and NADH as cofactor. Cyclic enones with methyl substitutions on the Cβ position (**1d**, **1e**) were not reduced by either *Dr*ER or *Rm*ER, similar to other members of this group such as YqjM and *Ts*ER. The only enal tested, citral (**3**), has a methyl group on the Cβ and reactivity was only detected for *Dr*ER. 2-Methylcyclopentenone (**1c**) and ketoisophorone (**1f**) were reduced by both *Dr*ER and *Rm*ER, albeit marginally, except for *Dr*ER which reduced approximately 75%

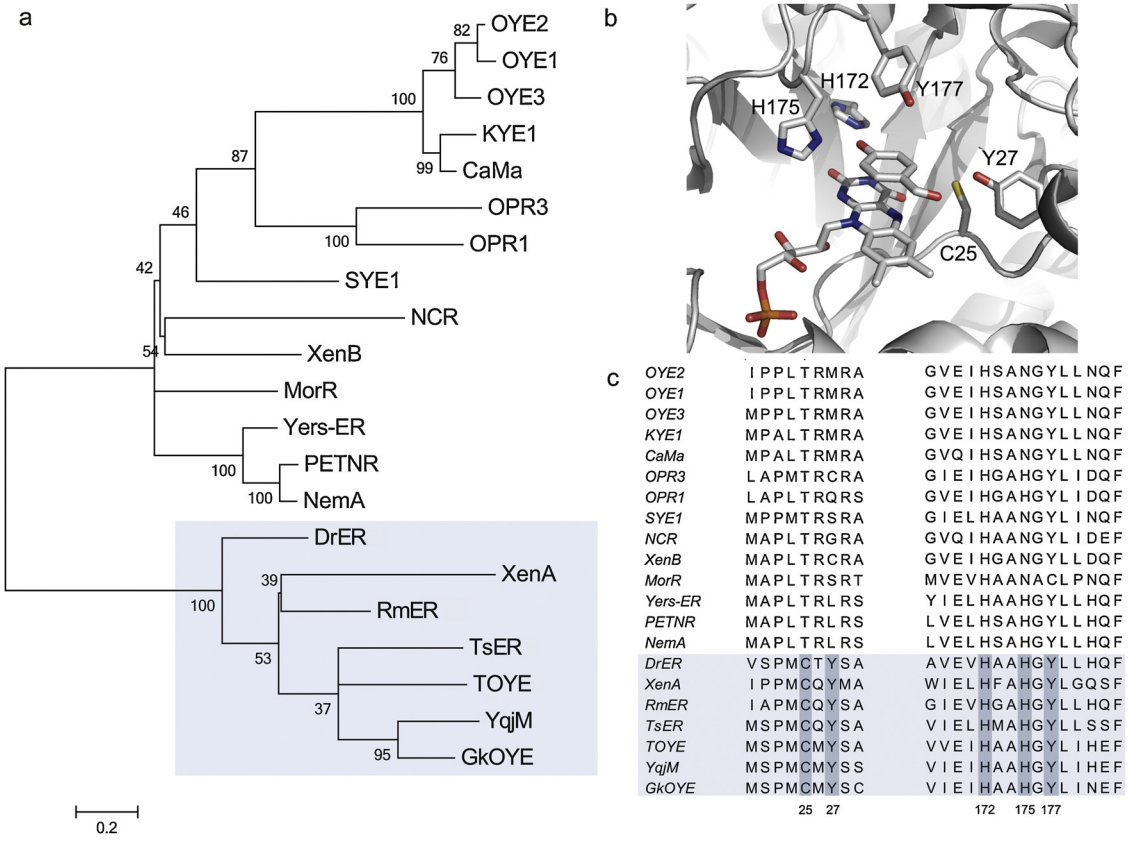


Fig. 1. (a) Phylogenetic relationship of *Dr*ER and *Rm*ER with previously cloned and characterized ene-reductases/OYEs through a bootstrap consensus un-rooted maximum-likelihood (ML) tree inferred from the WAG+I+G model. (b) Catalytic site of *Ts*ER with *p*-hydroxybenzaldehyde bound (PDB ID: 3GHJ) above the flavin mononucleotide (FMN) showing the conserved amino acids involved in catalysis and substrate binding. (c) Multiple sequence alignment of the ERs used for comparison with NCBI accession numbers given in parentheses. OYE2 and 3 *Saccharomyces cerevisiae* (AAA83386, AAA64522), OYE1 *Saccharomyces pastorianus* (*carlsbergensis;* Q02899), KYE1 *Kluyveromyces lactis* (AAA98815), CaMa *Candida macedoniensis* (BAD24850), OPR1 and 3 *Solanum lycopersicum* (AJ242551, NP_001233873), SYE1 *Shewanella oneidensis* (AAN55488), NCR *Zymomonas mobilis* (Q5NLA1), XenB *Pseudomonas fluorescens* (AF154062), MorR *Pseudomonas putdia* (AAC43569), Yers-ER *Yersinia bercovieri* (WP_005270358), PETNR *Enterobacter cloacae* (U68759), NemA *Escherichia coli* (BAA13186), *Dr*ER *Deinococcus radiodurans* (NP_295913), XenA *Pseudomonas putida* (AF154061), *Rm*ER *Ralstonia metallidurans* (YP_586990), *Ts*ER *Thermus scotoductus* (AM902709), *TO*YE *Thermoanaerobacter pseudethanolicus* (YP_001664021), YqjM *Bacillus subtilis* (BAA12619), *Gk*OYE *Geobacillus kaustophilus* (YP_148185). YqjM-like or "thermophilic-like" ERs are shown by shading in light grey. Dark grey shading indicates the catalytically important conserved amino acids. (For interpretation of the references to color in this text, the reader is referred to the web version of the article.)

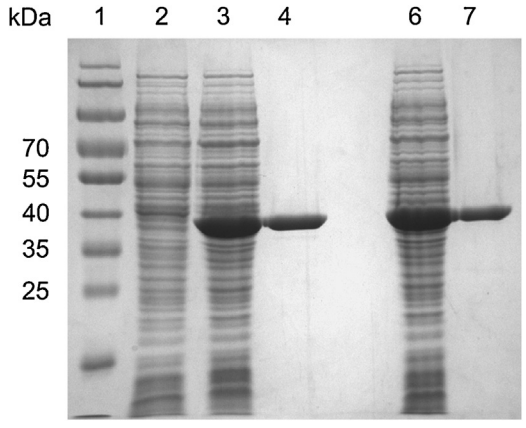


Fig. 2. SDS-PAGE analysis of the expression and purification of *Rm*ER and *Dr*ER. 1: Molecular weight marker, 2: soluble extract of *E. coli*, 3: soluble extract of *E. coli* expressing *Rm*ER, 4: purified *Rm*ER, 6: soluble extract of *E. coli* expressing *Dr*ER, 7: purified *Dr*ER.

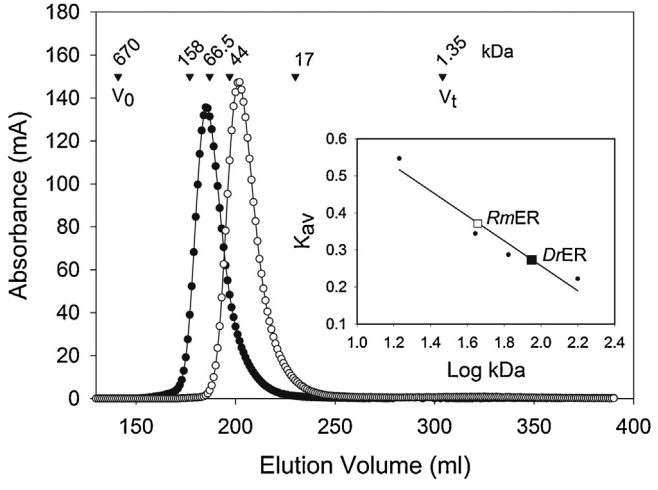


Fig. 3. Elution profile from size-exclusion chromatography of *Dr*ER (●) and *Rm*ER (○). Reference standards are shown as triangles with molecular weights in kDa. Inset: calibration curve, bovine γ-globulin (158 kDa), bovine serum albumin (66.5 kDa), chicken ovalbumin (44 kDa) and horse myoglobin (17 kDa). $K_{av} = (V_e - V_0)/(V_t - V_0)$.

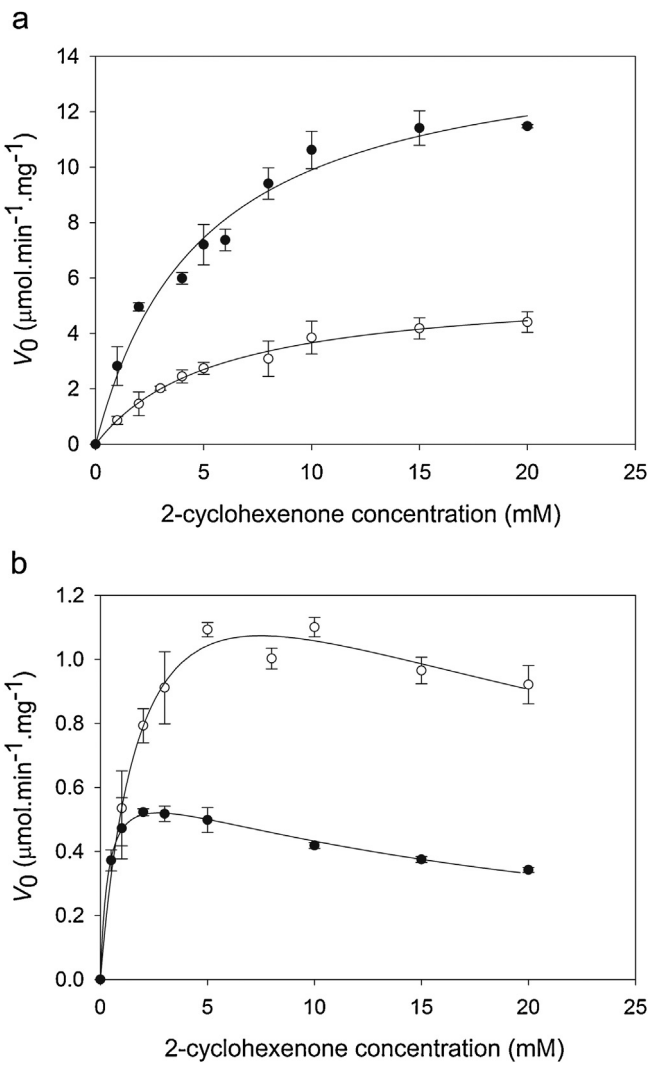
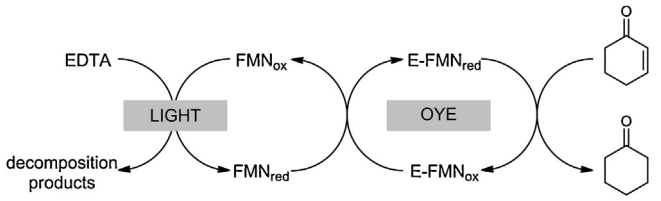


Fig. 4. Steady-state kinetics of *Dr*ER (●) and *Rm*ER (○) using varying concentrations of 2-cyclohexenone and 0.3 mM NADPH (**a**) or NADH (**b**). Error bars indicate standard deviation.

of the ketoisophorone within 5 h. The dimethyl α,β-unsaturated esters, dimethylfumarate (**2a**) and dimethylmaleate (**2b**), also served as substrates for both *Dr*ER and *Rm*ER, with the *cis* configuration the preferred substrate in both cases. Structurally more demanding substrates such as the isomers of carvone (**4**), proved to be converted by both *Dr*ER and *Rm*ER. The (*R*)-(−)-carvone (**4a**) was slightly preferred by both *Dr*ER and *Rm*ER. Apart from 2-cyclohexenone, 2-methyl-N-phenylmaleimide (**5a**) and its unsubstituted counterpart N-phenylmaleimide (**5b**), were the only substrates being completely converted after 5 h by both *Dr*ER and *Rm*ER.

The enantioselectivity of the ERs was evaluated using the isomers of carvone (**4**), ketoisophorone (**1f**), and 2-methylcyclopentenone (**1c**). Both ERs showed high enantioselectivity, forming 2*R*,5*S*-dihydrocarvone (>93% *de*) and 2R,5R-dihydrocarvone (>95% *de*) from R-(−)-carvone (**4a**) and S-(+)-carvone (**4b**) respectively. Similar to other members of this subgroup of ERs, ketoisophorone (**1f**) and 2-methylcyclopentenone (**1c**) were converted to their (*R*)- and (*S*)-enantiomeric products respectively. The low optical purity of (6*R*)-levodione (product of **1f**) has been attributed to the racemization of the product in aqueous solutions [45], likewise we noted a decrease in optical purity to approximately 90% *ee* after 24 h with both *Rm*ER and



Scheme 1. Schematic representation of the light driven cofactor regeneration pathway for the reduction of conjugated C=C double bonds. E-FMN represents the enzyme bound flavin group.

*Dr*ER. Moreover, multiple binding conformations have been shown to influence enantiopurity, and in the case of the reduction of 2-methylcyclopentenone, a "flipped" binding orientation [32] relative to cyclohexenone derivatives accounts for the reversal of the stereochemical outcome, yielding the *S*-enantiomer.

3.4. Cofactor regeneration

One of the major drawbacks still in the application of ERs is the requirement for expensive co-factors. Most researchers therefore use whole-cell systems, which often give low yields and enantioselectivies due to the interfering native ene-reductases or alcohol dehydrogenases [9,46]. Various co-factor regeneration systems are therefore being employed to overcome this limitation not only for ERs but also redox enzymes in general [47].

Enzymatic co-factor regeneration systems such as glucose dehydrogenase, glucose-6-phosphate dehydrogenase and formate dehdrogenase have been shown to affect yield and enantiopurity of products [48–50]. An alternative to these conventional enzymatic cofactor systems is the use of light-driven direct regeneration [51,52]. Previous attempts to use the light-driven cofactor regeneration system with *Ts*ER were problematic due to the fact that EDTA, which is used as sacrificial electron donor, binds calcium and magnesium which *Ts*ER requires for activity. We therefore tested *Dr*ER and *Rm*ER for their applicability with this system using 2-cyclohexenone (**1a**) as model substrate (Scheme 1).

Complete conversion of 5 mM 2-cyclohexanone was obtained with both *Dr*ER and *Rm*ER using the light-driven cofactor recycling (Fig. 5). Although initial kinetic characterization of the two ERs showed the *Dr*ER to have a higher specific rate with 2-cyclohexenone using NADPH as electron donor, surprisingly *Rm*ER was able to catalyze the complete conversion within 5 h, showing nearly twice the reaction rate observed with *Dr*ER. The effect

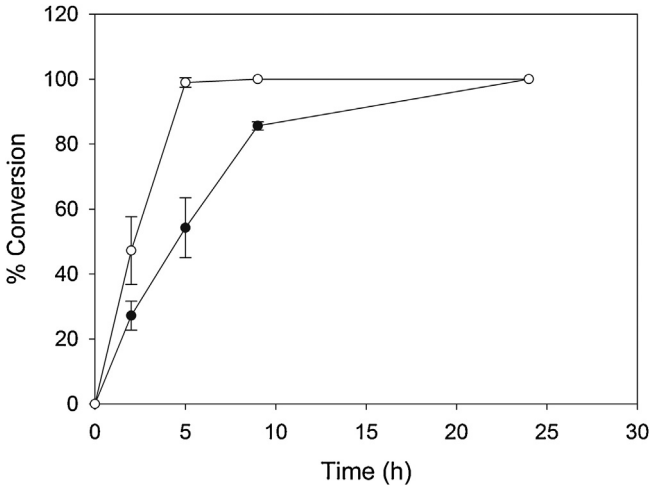


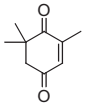
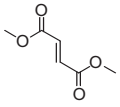
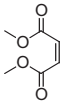
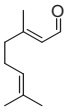
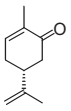
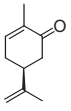
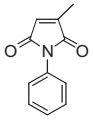
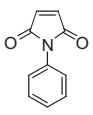
Fig. 5. Time course of 2-cylcohexenone reduction using *Dr*ER (●) and *Rm*ER (○) with the light driven cofactor regeneration system.

Table 1
Catalytic parameters of purified *Dr*ER and *Rm*ER towards 2-cyclohexenone with NADPH as electron donor.

	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
*Dr*ER	14.77 ± 0.60	4.92 ± 0.53	10.28	2.09
*Rm*ER	5.49 ± 0.35	5.51 ± 0.88	3.87	0.70

Conditions: NADPH = 0.3 mM, *Rm*ER (13 μg/ml) and *Dr*ER (6 μg/ml), 20 mM MOPS-NaOH (pH 7.4) containing 0.1 M NaCl at 30 °C.

Table 2
Substrate scope and selectivity of *Dr*ER and *Rm*ER.

Substrate		Conversion[a] (%)		Selectivity (*ee*/*de*) (%)	
		*Dr*ER	*Rm*ER	*Dr*ER	*Rm*ER
1a		>99	>99		
1b		73.5	62.8		
1c		8.8	6.5	58% (*S*)	>99% (*S*)
1d		n.c.	n.c.		
1e		n.c.	n.c.		
1f		75.3	3.0	99% (*R*)	97% (*R*)
2a		45.6	20.9		
2b		93.9	52.0		
3		8.5	n.c.	n.d.	
4a		99.0	83.1	95% (2*R*,5*R*)	96% (2*R*,5*R*)
4b		92.1	56.0	93% (2*R*,5*S*)	93% (2*R*,5*S*)
5a		>99	>99	n.d.	n.d.
5b		>99	>99		

n.c. No conversion detected after 5 h conversion.
n.d. Not determined.
[a] Percentage conversion determined by product to substrate ratios of GC analysis. All substrate concentrations were 1 mM, 2 mM NADH, 20 μg/mL ER, 5 h at 30 °C in 20 mM MOPS pH 7.4 with 0.1 M NaCl.

Table 3
Specific activities of *Dr*ER and *Rm*ER.

Substrate	Specific activity (μmol min^{-1} mg^{-1})	
	*Dr*ER	*Rm*ER
Molecular oxygen	0.69 ± 0.06	0.70 ± 0.05
1a	11.90 ± 0.18	2.86 ± 0.10
FAD	1.43 ± 0.04	9.43 ± 0.63
FMN	2.75 ± 0.25	13.66 ± 1.11
FAD and **1a**	11.94 ± 1.08	9.05 ± 0.07
FMN and **1a**	11.90 ± 0.43	10.90 ± 0.08

Conditions: **1a** = 5 mM, NADPH, FAD, FMN = 0.3 mM, *Dr*ER = 10 μg/mL, *Rm*ER = 5 μg/mL, 30 °C in 20 mM MOPS pH 7.4 with 0.1 M NaCl, 5% v/v ethanol, 5 mM EDTA.

of FMN and EDTA on the ERs were consequently investigated and revealed both FMN and FAD to be excellently accepted by *Rm*ER compared to *Dr*ER. This might be due to the observed differences in the quaternary structure of the ERs as the neighbouring subunits of multimeric ERs have been shown to partially cover the active site opening, possibly obstructing access for lager substrates (Table 3).

Finally, the influence of the co-factor regeneration system on asymmetric reduction was determined by using (*R*)-(−)-carvone (**4a**) as substrate. Gratifyingly the light-driven system yielded essentially identical optical purities (97% *ee*) to that observed in the NADPH reactions.

4. Conclusions

Two new ene-reductases from *D. radiodurans* and *R. metallidurans*, both belonging to the YqjM-like subclass of OYEs were cloned, successfully expressed in *E. coli* and purified. Testing of different substrate classes revealed slight differences in the two ERs catalytic preferences and enantioselectivies. For most of the substrates tested, the specific activity of *Dr*ER was greater than that of *Rm*ER. *Rm*ER also showed only minimal conversion of ketoisophorone and no activity towards citral, whereas *Dr*ER readily converted ketoisophorone and also conversion of citral was observed. NAD(P)H free conversion of 2-cyclohexenone, as well as asymmetric reduction of (*R*)-(−)-carvone was achieved using both *Dr*ER and *Rm*ER through a light-driven co-factor regenerations system using EDTA as electron donor. Crystallization for X-ray structure determination is currently underway in our laboratories to gain insight into catalytic differences observed between *Dr*ER and *Rm*ER as well as with other members of this subclass. The effect of oligomerization on activity will also be investigated.

Acknowledgements

This work was supported by the National Research Foundation South Africa and the Oppenheimer Memorial Trust. The authors thank Maarten Gorseling, Remco van Oosten (Delft University of Technology, NL) and Sarel Marais (University of the Free State, SA) for GC and HPLC analysis.

References

[1] O. Warburg, W. Christian, Naturwissenschaften 20 (1932) 980–981.
[2] P.A. Karplus, K.M. Fox, V. Massey, FASEB J. 9 (1995) 1518–1526.
[3] K. Stott, K. Saito, D.J. Thiele, V. Massey, J. Biol. Chem. 268 (1993) 6097–6106.
[4] D.J. Bougioukou, J.D. Stewart, in: K. Drauz, H. Gröger, O. May (Eds.), Enzyme Catalysis in Organic Synthesis, third ed., Wiley-VCH, Weinheim, 2012, pp. 1111–1163.
[5] H.S. Toogood, J.M. Gardiner, N.S. Scrutton, ChemCatChem 2 (2010) 892–914.
[6] A.D.N. Vaz, S. Chakraborty, V. Massey, Biochemistry 34 (1995) 4246–4256.
[7] R.M. Kohli, V. Massey, J. Biol. Chem. 273 (1998) 32763–32770.
[8] H.L. Messiha, N.C. Bruce, B.M. Sattelle, M.J. Sutcliffe, A.W. Munro, N.S. Scrutton, J. Biol. Chem. 280 (2005) 27103–27110.
[9] R. Stuermer, B. Hauer, M. Hall, K. Faber, Curr. Opin. Chem. Biol. 11 (2007) 203–213.
[10] H.-U. Blaser, B. Pugin, F. Spindler, Top. Organomet. Chem. 42 (2012) 65–102.
[11] C.K. Winkler, G. Tasnádi, D. Clay, M. Hall, K. Faber, J. Biotechnol. 162 (2012) 381–389.
[12] C. Stueckler, M. Hall, H. Ehammer, E. Pointner, W. Kroutil, P. Macheroux, K. Faber, Org. Lett. 9 (2007) 5409–5411.
[13] T.B. Fitzpatrick, N. Amrhein, P. Macheroux, J. Biol. Chem. 278 (2003) 19891–19897.
[14] J.F. Chaparro-Riggers, T.A. Rogers, E. Vazquez-Figueroa, K.M. Polizzi, A.S. Bommarius, Adv. Synth. Catal. 349 (2007) 1521–1531.
[15] Y. Meah, V. Massey, Proc. Natl. Acad. Sci. USA 97 (2000) 10733–10738.
[16] H.S. Toogood, A. Fryszkowska, V. Hare, K. Fisher, A. Roujeinikova, D. Leys, J.M. Gardiner, G.M. Stephens, N.S. Scrutton, Adv. Synth. Catal. 350 (2008) 2789–2803.
[17] T.M. Barna, H. Khan, N.C. Bruce, I. Barsukov, N.S. Scrutton, P.C. Moody, J. Mol. Biol. 310 (2001) 433–447.
[18] H. Nivinskas, J. Sarlauskas, Z. Anusevicius, H.S. Toogood, N.S. Scrutton, N. Cenas, FEBS J. 275 (2008) 6192–6203.
[19] Y. Meah, B.J. Brown, S. Chakraborty, V. Massey, Proc. Natl. Acad. Sci. USA 98 (2001) 8560–8565.
[20] C.E. French, S. Nicklin, N.C. Bruce, J. Bacteriol. 178 (1996) 6623–6627.
[21] K.M. Fox, P.A. Karplus, Structure 2 (1994) 1089–1105.
[22] C. Breithaupt, J. Strassner, U. Breitinger, R. Huber, P. Macheroux, A. Schaller, T. Clausen, Structure 9 (2001) 419–429.
[23] C. Breithaupt, R. Kurzbauer, H. Lilie, A. Schaller, J. Strassner, R. Huber, P. Macheroux, T. Clausen, Proc. Natl. Acad. Sci. USA 103 (2006) 14337–14342.
[24] T. Barna, H.L. Messiha, C. Petosa, N.C. Bruce, N.S. Scrutton, P.C.E. Moody, J. Biol. Chem. 277 (2002) 30976–30983.
[25] J.J. Griese, R.P. Jakob, S. Schwarzinger, H. Dobbek, J. Mol. Biol. 361 (2006) 140–152.
[26] K. Kitzing, T.B. Fitzpatrick, C. Wilken, J. Sawa, G.P. Bourenkov, P. Macheroux, T. Clausen, J. Biol. Chem. 280 (2005) 27904–27913.
[27] D. van den Hemel, A. Brigé, S.N. Savvides, J. Van Beeumen, J. Biol. Chem. 281 (2006) 28152–28161.
[28] M. Schittmayer, A. Glieder, M.K. Uhl, A. Winkler, S. Zach, J.H. Schrittwieser, W. Kroutil, P. Macheroux, K. Gruber, S. Kambourakis, J.D. Rozzell, M. Winkler, Adv. Synth. Catal. 353 (2011) 268–274.
[29] D.J. Opperman, B.T. Sewell, D. Litthauer, M.N. Isupov, J.A. Littlechild, E. van Heerden, Biochem. Biophys. Res. Commun. 393 (2010) 426–431.
[30] B.V. Adalbjörnsson, H.S. Toogood, A. Fryszkowska, C.R. Pudney, T.A. Jowitt, D. Leys, N.S. Scrutton, ChemBioChem 11 (2010) 197–207.
[31] G. Oberdorfer, G. Steinkellner, C. Stueckler, K. Faber, K. Gruber, ChemCatChem 3 (2011) 1562–1566.
[32] S.K. Padhi, D.J. Bougioukou, J.D. Stewart, J. Am. Chem. Soc. 131 (2009) 3271–3280.
[33] A.Z. Walton, W.C. Conerly, Y. Pompeu, B. Sullivan, J.D. Stewart, ACS Catal. 1 (2011) 989–993.
[34] D.J. Bougioukou, S. Kille, A. Taglieber, M.T. Reetz, Adv. Synth. Catal. 351 (2009) 3287–3305.
[35] F.W. Studier, Protein Expr. Purif. 41 (2005) 207–234.
[36] U.K. Laemmli, Nature 227 (1970) 680–685.
[37] C.E. Paul, S. Gargiulo, D.J. Opperman, I. Lavandera, V. Gotor-Fernández, V. Gotor, A. Taglieber, I.W.C.E. Arends, F. Hollmann, Org. Lett. 15 (2013) 180–183.
[38] M.S. van Dyk, E. van Rensburg, I.P.B. Rensburg, N. Moleleki, J. Mol. Catal. B Enzym. 5 (1998) 149–154.
[39] R.C. Edgar, Nucleic Acids Res. 32 (2004) 1792–1797.
[40] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, Mol. Biol. Evol. 28 (2011) 2731–2739.
[41] S. Whelan, N. Goldman, Mol. Biol. Evol. 18 (2001) 691–699.
[42] D.J. Opperman, L.A. Piater, E. van Heerden, J. Bacteriol. 190 (2008) 3076–3082.
[43] O. Spiegelhauer, T. Werther, S. Mende, S.H. Knauer, H. Dobbek, J. Mol. Biol. 403 (2010) 286–298.
[44] T.B. Fitzpatrick, S. Auweter, K. Kitzing, T. Clausen, N. Amrhein, P. Macheroux, Protein Expres. Purif. 36 (2004) 280–291.
[45] A. Fryszkowska, H. Toogood, M. Sakuma, J. Gardiner, G. Stephens, N. Scrutton, Adv. Synth. Catal. 351 (2009) 2976–2990.
[46] M. Hall, B. Hauer, R. Stuermer, W. Kroutil, K. Faber, Tetrahedron: Asymmetry 17 (2006) 3058–3062.
[47] F. Hollmann, I.W.C.E. Arends, K. Buehler, ChemCatChem 2 (2010) 762–782.
[48] M. Hall, C. Stueckler, W. Kroutil, P. Macheroux, K. Faber, Angew. Chem. Int. Ed. 46 (2007) 3934–3937.
[49] M. Hall, C. Stueckler, H. Ehammer, E. Pointner, G. Oberdorfer, K. Gruber, B. Hauer, R. Stuermer, W. Kroutil, P. Macheroux, K. Faber, Adv. Synth. Catal. 350 (2008) 411–418.
[50] M. Hall, C. Stueckler, B. Hauer, R. Stuermer, T. Friedrich, M. Breuer, W. Kroutil, K. Faber, Eur. J. Org. Chem. 2008 (2008) 1511–1516.
[51] A. Taglieber, F. Schulz, F. Hollmann, M. Rusek, M.T. Reetz, ChemBioChem 9 (2008) 565–572.
[52] M.M. Grau, J. van Der Toorn, L. Otten, P. Macheroux, A. Taglieber, F. Zilly, I.W.C.E. Arends, F. Hollmann, Adv. Synth. Catal. 351 (2009) 3279–3286.