Journal of Molecular Catalysis B: Enzymatic 115 (2015) 1–7

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Exploring the synthetic applicability of a new carboxylic acid reductase from *Segniliparus rotundus* DSM 44985

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ARTICLE INFO

Article history:
Received 11 November 2014
Received in revised form 26 January 2015
Accepted 26 January 2015
Available online 7 February 2015

Keywords:
Carboxylic acids
Biocatalysis
Reduction
Chemoselectivity

ABSTRACT

A new carboxylic acid reductase (CAR) gene from *Segniliparus rotundus* DSM 44985 was overexpressed in *Escherichia coli*. The recombinant enzyme exhibited high activity toward a variety of aromatic and aliphatic carboxylic acids. Especially, it effectively reduced 4-hydroxybenzoic acid (**8a**) and 4-nitrobenzoic acid (**19a**), toward which the known *Nocardia* CAR exhibited no or little activity. The recombinant *E. coli* cells co-expressing the *Segniliparus* CAR and *Nocardia* PPTase genes catalyzed the reductions of vanillic acid (**20a**) and 3,4-dihydroxyphenylacetic acid (**25a**) to give vanillyl alcohol (**20c**) and 3-hydroxytyrosol (**25c**) with high yield, respectively. The endogenous aldehyde reductases of *E. coli* should be responsible for the further reduction of the produced aldehydes. These results demonstrated that *Segniliparus* CAR was a useful addition to the biocatalyst tool-box for the reduction of carboxylic acids and might find applications in the synthesis of valuable bio-based chemicals from renewable resources.

1. Introduction

Carboxylic acids such as fatty acids, ferulic acid, and many others are available in abundance from renewable resources. As economic precursors, they can be used to prepare the bio-based products [1]. For example, fatty acids are reduced to fatty alcohols, which can be used as biofuels and intermediates in synthesizing detergents, surfactants, and polymers [2]. The reduction of carboxylic acids is a useful and challenging task. Chemical methods frequently depend on activated carboxylates by $LiAlH_4$, $NaBH_4$, and their derivatives as reducing agents [3], which are unfavorable for large scale applications from the environmental and safety points of view. More importantly, it is difficult to achieve the chemoselective reduction of carboxylic acids by chemical methods, because other reducible moieties such as carbonyl group and C=C bond can be easily affected by such strong reducing agents [4]. On the contrary, biocatalytic reductions of carboxylic groups are often highly selective reactions under mild conditions, thus overcoming the difficulties mentioned above. Furthermore, enzymatic reactions are generally appreciated by producers of pharmaceuticals and fine chemicals, and also biocatalytic reduction processes are gaining increased attention [5]. In the past 20 years, whole-cell bioreductions of carboxylic acids by a number of microorganisms have been reported, including *Corynespora melloni* [6], *Coriolus* [6], *Nocardia* sp. [7], *Pyrococcus furiosus* [8,9], and some plant cells (*Actinidia chinensis*, *Convolvulus sepium*, *Daucus carota*, and so on) [10]. Although many potential sources for carboxylic acid reductases (CAR) have been reported [1], only a few CARs have been biochemically characterized, including those from *Nocardia* [7,11], *Mycobacterium* [12], *Streptomyces griseus* [12], *Mycobacterium marinum* [13], and subtype II nonribosomal peptide synthetase (NRPS)-like proteins such as lnaA [14] and ATEG_03630 [15]. In addition, two reversible aldehyde oxidoreductases, which not only oxidize aldehydes but also reduce carboxylic acids, were identified [16–19]. These CARs possess the following consensus sequence characteristics: (i) their N-terminals and C-terminals show significant sequence similarity to AMP-binding proteins and NAD(P)-dependent aldehyde dehydrogenases, respectively, (ii) they all have a phosphopantetheine attachments site. CAR requires post-translational phosphopantetheinylation catalyzed by phosphopantetheine transferases (PPTase) [20]. ATP, Mg^{2+}, and NADPH as cofactors are necessary for the reduction [11,20], and ATP and NADPH are consumed in stoichiometric amounts (Fig. 1) [21]. Meanwhile, it has been found that two genes in the grixazone biosynthesis gene cluster constituted a CAR [22]. The substrate profile of an enzyme provides guidance for its synthetic application, but this important information of the known CARs is limited [12,13,23,24].

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http://dx.doi.org/10.1016/j.molcatb.2015.01.014
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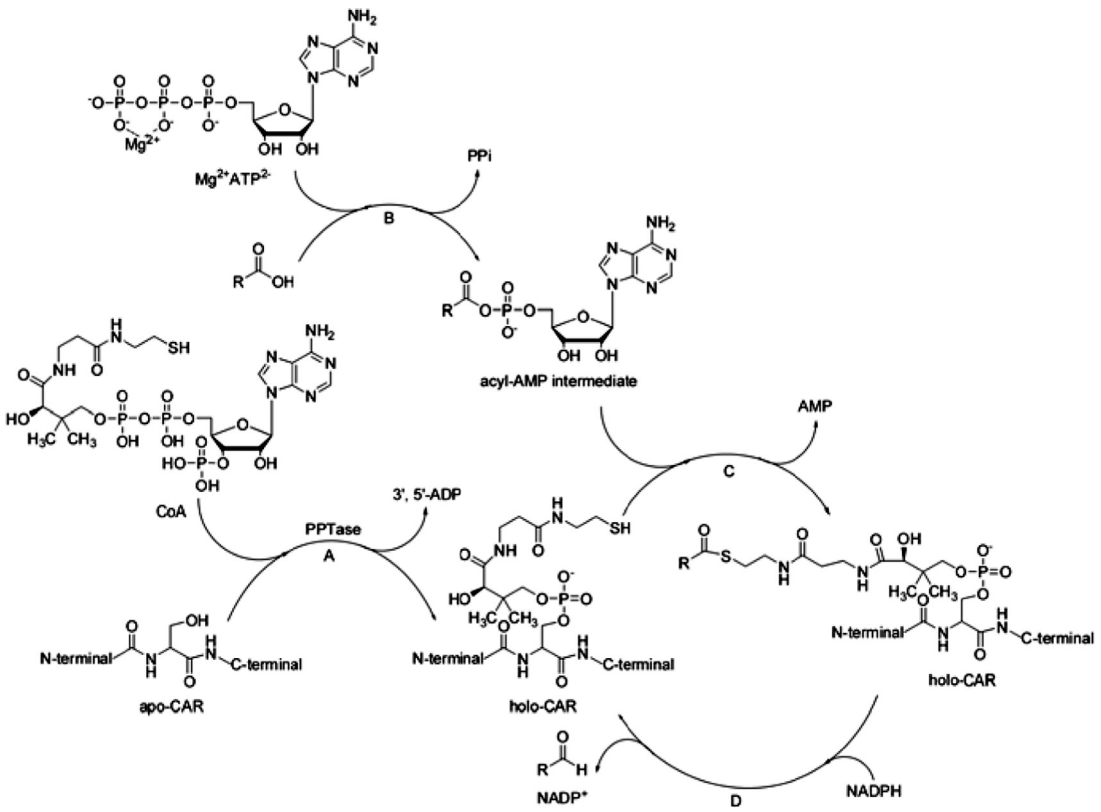


Fig. 1. Catalytic cycle of carboxylic acid reduction by CAR [20]. (A) Transfer of a phosphopantetheine moiety from the donor (CoA) to an acceptor apo-CAR conserved serine residue catalyzed by PPTase. (B) Adenylation of acid at the N terminus by ATP yielding acyl-AMP intermediate. (C) Nucleophilic attack of the phosphopantetheine thiol at the carbonyl carbon of acyl-AMP intermediate, releasing AMP and forming covalently bound acyl thioester. (D) The benzoyl thioester-phosphopantetheinyl "arm" swings from the adenylating domain to the reduction domain, leading to the reduction of thioester by NADPH, releasing aldehyde, $NADP^+$, and free holo-CAR ready for the next reduction cycle.

Therefore, in an effort to establish a tool-box of carboxylic acid reductases with instructive information, a new carboxylic acid reductase gene from *Segniliparus rotundus* DSM 44985 was selected by NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) using the *Nocardia* CAR (accession number AAR91681.1) as template. The *Segniliparus* CAR effectively catalyzed the reduction of a series of aromatic and aliphatic carboxylic acids including 4-hydroxybenzoic acid (**8a**) and 4-nitrobenzoic acid (**19a**), which were not reduced by the known *Nocardia* CAR. The *Segniliparus* CAR gene was thus co-expressed with a *Nocardia* PPTase gene in *Escherichia coli*. The resulting whole-cell biocatalyst was evaluated for the reduction of carboxylic acids without adding the expensive cofactors, in an effort to exploring its synthetic applicability.

2. Materials and methods

2.1. Materials

PrimeSTAR HS DNA polymerase and the DNA ligation kit were from TaKaRa (Dalian, China). FastDigest Restriction Enzymes were purchased from Fermentas (Shenzhen, China). Vectors [pET32a(+) and pETDuet-1] were purchased from Novagen (Schwalbach, Germany). The plasmid extraction kit was from CWBIO (Beijing, China). The gel extraction kit was from Tiangen Biotech (Beijing, China). The lyophilized powder of glucose dehydrogenase (GDH) from *Bacillus subtilis*, with a specific activity of 1.1 U/mg, was prepared in our laboratory. All carboxylic acids were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), Alfa Aesar (Shanghai, China) or Sigma–Aldrich (St. Louis, USA). ^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE-III 500 or 600 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany).

2.2. Expression and purification of CAR and PPTase

The optimized N-terminal His-tagged *Segniliparus* CAR and *Nocardia* PPTase genes were synthesized and cloned into the *Nde*I/*Xho*I sites of pET32a(+), respectively, by Shanghai Xuguan Biotechnological Development Co., Ltd. (Shanghai, China). The resulting plasmids [pET32a(+)-CAR and pET32a(+)-PPTase] were transformed into *E. coli* BL21 (DE3), respectively. A culture of *E. coli* BL21 (DE3) cells harboring pET32a(+)-CAR or pET32a(+)-PPTase was grown overnight in LB-ampicillin (100 μg/mL) medium (5 mL) at 37 °C, and then inoculated into 1 L of LB-ampicillin (100 μg/mL) medium. The resulting culture was incubated continually at 200 rpm in a rotary shaker at 37 °C until cells reach mid-log growth (OD_{600} of 0.5–1.0), which was followed by the addition of 0.5 mM IPTG and further incubation for 12 h at 25 °C. Cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4 °C, and disrupted by high pressure homogenizer after re-suspension in binding buffer (20 mM sodium phosphate buffer, 0.5 M NaCl, 20 mM imidazole, pH 7.4). His-CAR or His-PPTase fusion protein in the supernatant fraction was collected from the crude cell lysate by centrifugation at 12,000 × *g* for 20 min. Protein purification was performed on a HisTrap™ FF crude column (GE Healthcare, Piscataway, USA), and the protein was desorbed with an elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). The purified proteins His-CAR or His-PPTase were dialyzed in a sodium phosphate buffer (50 mM, pH 7.5) and then stored at −20 °C for further use.

2.3. Molecular weight of Segniliparus CAR

The molecular mass of *Segniliparus* CAR was determined by gel filtration chromatography using a Superdex 200 10/300 GL column and the high-molecular-weight gel filtration calibration kit (GE Healthcare, Piscataway, USA) was used for calibration. The sodium phosphate buffer (50 mM, pH 7) containing 150 mM NaCl was used as the eluent. The flow rate was 0.4 mL/min and the absorbance at 280 nm was monitored.

2.4. Standard reduction procedure

The His-CAR (1.5 mg) was incubated with His-PPTase (295 μg) in the presence of CoA (1 mM) as a cofactor for 1 h at 28 °C in a final volume of 600 μL of sodium phosphate buffer (100 mM, pH 7.5) containing 10 mM of $MgCl_2$. The resulting enzyme mixture (holo-CAR, 50 μg) was mixed with $NADP^+$ (0.9 mM), GDH (1 U, one unit corresponds to the amount of enzyme which could reduce 1 μmol $NADP^+$ to NADPH per minute), glucose (60 mM), $MgCl_2$ (10 mM), substrate (5 or 10 mM, from 1 M stock solution in DMSO), and ATP (15 mM) in Tris–HCl buffer (100 mM, pH 9) with a final volume of 1 mL. The reaction mixture was incubated at 100 rpm in a rotary shaker at 35 °C for 16 h, and extracted with 1 mL of ethyl acetate after the pH was adjusted to 2–3 with 1 M HCl solution. The organic extracts were dried over anhydrous sodium sulfate and analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) to determine the amount of substrate (**a**) and products (aldehyde **b**, alcohol **c**) in the mixture. All experiments were conducted in triplicate.

2.5. Determination of kinetic parameters

The enzyme mixtures were prepared by following the standard assay procedure. The reaction was performed in Tris–HCl buffer (100 mM, pH 9) containing enzyme mixture (holo-CAR, 20 μL, 5.87 μg), $MgCl_2$ (10 mM), varying concentrations of benzoic acid (**5a**, 0.05–15 mM), ATP (1.2 mM), and NADPH (0.4 mM) in a final volume of 200 μL. For ATP, **5a** (4 mM), NADPH (0.4 mM), enzyme mixture (holo-CAR, 20 μL, 5.87 μg) and ATP in the range of 0.01–0.5 mM were used for the activity assay. For NADPH, **5a** (4 mM), ATP (1.2 mM), enzyme mixture (holo-CAR, 20 μL, 5.87 μg) and NADPH in the range of 0.01–0.5 mM were used for the activity assay. The activity of CAR was determined by spectrophotometrically measuring the oxidation of NADPH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). The specific activity was defined as the number of μmol of NADPH converted in 1 min by 1 mg of enzyme ($\mu mol\ min^{-1}\ mg^{-1}$). All experiments were conducted in triplicate. The kinetic parameters were obtained by measuring the initial velocities of the enzymatic reaction and curve-fitting according to the Michaelis–Menten equation using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA).

2.6. Mechanism of Segniliparus CAR

To determine whether the mechanism is consistent with that of the *Nocardia* CAR (Fig. 1), the following reaction mixtures of sodium phosphate buffer (1 mL, 100 mM, pH 7.5) were incubated at 30 °C or 35 °C for 12 h (Supplementary Fig. S2 and Table T1). For the trapping of possible intermediate phenylacetyl thioester in Fig. 1, the reaction mixture contained enzyme mixture (holo-CAR, 100 μg), phenylacetic acid (**23a**, 10 mM) from 1 M stock solution in methanol (Supplementary Fig. S2a) or DMSO (Supplementary Fig. S2b), $NADP^+$ (0.9 mM), GDH (1 U), glucose (60 mM), ATP (15 mM) and $MgCl_2$ (10 mM). For the effect of Mg^{2+} and post-translational phosphopantetheinylation, the reaction mixture contained His-CAR (apo-CAR) or enzyme mixture (holo-CAR) (30 μg), **23a** (10 mM, from 1 M stock solution in DMSO), $NADP^+$ (0.9 mM), GDH (1 U), glucose (60 mM), ATP (15 mM) and $MgCl_2$ (0 mM or 10 mM) (Supplementary Table T1).

2.7. Substrate specificity

The reduction of a series of carboxylic acids was carried out by following the standard reduction procedure. The yields were determined by GC analysis and the products were characterized by GC–MS.

2.8. Analytical procedure

GC was performed using a CP-Chirasil-DEXCB column (30 m × 0.25 mm × 0.25 μm) with flame ionization detector. The carrier gas was helium at a flow rate of 2 mL/min. The column temperature was controlled as follows: 120 °C – 10 °C/min – 160 °C (8 min) – 40 °C/min – 180 °C (5 min). The identity of products was confirmed with authentic standards. The retention times for phenylacetaldehyde (**23b**), phenethyl alcohol (**23c**) and **23a** were 3.2, 4.7 and 9.0 min, respectively. Yield was expressed as a molar ratio of product to product plus substrate determined by GC. GC–MS was performed using an Agilent 7890 GC, equipped with a HP-5 capillary column (30 m × 0.25 mm × 0.25 μm) and a mass spectrometer 5975C as detector. The carrier gas was helium at a flow rate of 1 mL/min. Column temperature was controlled as follows: 60 °C (4 min) – 20 °C/min – 200 °C (6 min) – 20 °C/min – 250 °C (4 min). For MS detection, the electron ionization was performed with an ionization energy of 70 eV. The products were identified by comparing the fragmentation patterns with the NIST mass spectral library.

2.9. Preparation of E. coli cells co-expressing CAR and PPTase

A CAR DNA fragment was acquired from the vector pET32a(+)-CAR by digesting at the restriction sites *Nde*I and *Xho*I, and then ligated by T4 DNA ligase into pETDuet-1 at the same restriction sites to generate the expression vector pETDuet-1-CAR. The PPTase gene was amplified by PCR using plasmid pET32a(+)-PPTase as template and primers containing the restriction sites *Nco*I and *Sal*I, respectively, PPTase-F 5′-CATGCCATGGCAATGATCGAAACCATCCTGCCG-3′ and PPTase-R 5′-ACGCGTCGACTTATTAAGCGTAAGCGATCGCGG-3′. The PCR fragment was digested with *Nco*I and *Sal*I, and then ligated with pETDuet-1-CAR at the same restriction sites to afford the expression vector pETDuet-1-PPTase-CAR, which was confirmed by DNA sequencing. The confirmed recombinant vector was transformed into *E. coli* BL21(DE3). A 100 mL of LB-ampicillin (100 μg/mL) medium harboring *E. coli* BL21(DE3)/pETDuet-1-PPTase-CAR was grown overnight and then inoculated into 9 L of LB-ampicillin (100 μg/mL) medium in a fermenter. When the cells reached the mid-log growth period (OD_{600} of 0.5–1.0), the induction was initiated by the addition of 0.5 mM IPTG and the culture was incubated for another 12 h at 25 °C. Cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4 °C, and washed once with phosphate buffer (100 mM, pH 8).

2.10. Reduction with whole cell biocatalyst

Glucose (22.2 mM), *E. coli* BL21(DE3)/pETDuet-1-PPTase-CAR (wet cells, 10 g), and the substrate (**20a**, 5.0 mM) were mixed in the sodium phosphate buffer (100 mL, 100 mM, pH 8). The resulting mixture was incubated at 200 rpm in a rotary shaker at 30 °C, and the reaction was monitored by GC. After 29 h, the pH of the reaction mixture was adjusted to 2–3 with 2 M HCl and the mixture was filtered through a Celite pad to remove the biomass. The resulting aqueous solution was extracted with ethyl acetate. The

organic phase was dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by a silica gel column to give the product.

*2.10.1. 4-Hydroxy-3-methoxybenzyl alcohol (**20c**)*

20c (53.8 mg, 0.35 mmol, 69%) was obtained from vanillic acid (**20a**). ^{1}H NMR (600 MHz, DMSO-d_6): δ = 8.75 (s, 1H, 4-OH), 6.87 (s, 1H, 2-H), 6.65–6.73 (m, 2H, 5-H, 6-H), 4.97 (t, J = 5.7 Hz, 1H, —CH_2OH), 4.37 (d, J = 5.5 Hz, 2H, —CH_2—), 3.75 (s, 3H, OCH_3). ^{13}C NMR (150 MHz, DMSO-d_6): δ = 147.34 (C-3), 145.27 (C-4), 133.47 (C-1), 119.07 (C-6), 115.01 (C-5), 111.03 (C-2), 62.97 (—CH_2OH), 55.49 (OCH_3).

*2.10.2. 2-(3,4-Dihydroxyphenyl)ethanol (**25c**)*

25c (32.9 mg, 0.21 mmol, 42%) was obtained from 3,4-dihydroxyphenylacetic acid (**25a**). ^{1}H NMR (500 MHz, DMSO-d_6): d = 8.70 (br. s., 1H, 3′-OH), 8.60 (br. s., 1H, 4′-OH), 6.59 (d, J = 8.3 Hz, 1H, 5′-H), 6.56 (d, J = 1.0 Hz, 1H, 2′-H), 6.41 (d, J = 7.8 Hz, 1H, 6′-H), 4.55 (br. s., 1H, 1-OH), 3.48 (br. s., 2H, 1-CH_2), 2.51 (t, J = 7.3 Hz, 2H, 2-CH_2). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 145.54 (C-3′), 143.97 (C-4′), 130.81 (C-1′), 120.11 (C-6′), 116.93 (C-2′), 116.02 (C-5′), 63.32 (C-1), 39.21 (C-2).

*2.10.3. 3-(3-Methoxy-4-hydroxyphenyl)-1-propanol (**29d**)*

29d (20.0 mg, 0.11 mmol, 22%) was obtained from *trans*-ferulic acid (**29a**). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.63 (s, 1H, 4′-OH), 6.71 (s, 1H, 2′-H), 6.63 (d, J = 7.8 Hz, 1H, 5′-H), 6.54 (d, J = 7.8 Hz, 1H, 6′-H), 4.43 (t, J = 5.1 Hz, 1H, 1-OH), 3.72 (s, 3H, —OCH_3), 3.39 (m, 2H, 1-CH_2), 2.43–2.50 (m, 2H, 3-CH_2), 1.65 (quin, J = 7.0 Hz, 2H, 2-CH_2). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 148.00 (C-3′), 144.98 (C-4′), 133.62 (C-1′), 120.95 (C-6′), 115.90 (C-5′), 113.11 (C-2′), 60.84 (C-1), 56.16 (OCH_3), 35.30 (C-2), 31.91 (C-3).

3. Results and discussion

3.1. Purification and biochemical characterization of Segniliparus CAR

A putative carboxylic acid reductase from *S. rotundus* DSM 44985 (accession number YP_003658971.1), which possessed the CAR consensus sequence characteristics, was chosen. The gene was synthesized and expressed in *E. coli* [11]. Since PPTase from *S. rotundus* has not been determined, a known *Nocardia* PPTase (accession number ABI83656.1) was selected for the post-translational phosphopantetheinylation of *Segniliparus* CAR [20]. Recombinant His-CAR and His-PPTase were produced as soluble proteins under the same conditions (OD_{600} – 0.6, 0.5 mM IPTG, 25 °C, 12 h) and purified in one chromatographic step using a HisTrap^{TM} FF crude column (Supplementary Fig. S1). The molecular mass of His-CAR was estimated to be about 158 kDa by gel filtration chromatography. Since its theoretical value is 129 kDa, this enzyme is a monomeric protein, which is consistent with *Nocardia* CAR [23].

The reaction mechanism of *Segniliparus* CAR was similar to that of *Nocardia* CAR [20,25]. When methanol was added into the reaction system (**23a** as substrate), methyl phenylacetate was observed by GC (Supplementary Fig. S2). The methyl ester was probably produced from the reaction of methanol with phenylacetyl thioester (Fig. 1). Similar aryl-aldehyde NADP$^+$ oxidoreductase from *Neurospora crassa* was demonstrated to catalyze a rapid exchange of pyrophosphoric acid (PPi) with ATP during the reduction of **5a** [26]. The adenylation of some carboxylic acids involving ATP to be converted to AMP and PPi was almost in equilibrium [26,27]. For *Nocardia* CAR, benzoyl-AMP had a significantly lower (67-fold) apparent K_m (9.66 ± 0.71 μM) and a higher (1.3-fold) V_{max} (7.50 ± 0.18 μmol min^{-1} mg^{-1}) than those for **5a** [23,25]. Therefore, the reactions B and C at the N terminus of CAR seem to be rapid compared to the rate of acid reduction D (Fig. 1), which may be the rate-limiting step in the whole reaction. The N-terminal domain of CAR is responsible for adenylation and thioesterification of carboxylic acid, and it may play a role in determining the substrate specificity, which is similar to the adenylation domain of NRPS [28]. No reaction was observed when apo-CAR was used in the reaction system (**23a** as substrate) or the reaction system did not contain Mg^{2+} (Supplementary Table T1), indicating that post-translational phosphopantetheinylation and Mg^{2+}, ATP, and NADPH as cofactors were necessary for this enzymatic reduction.

The optimum pH of the enzymatic reduction of **23a** was between pH 9 and 9.5, but yield of **23a** did not significantly decrease at pH 7–8 and kept at more than 80% (Supplementary Fig. S3). This is probably because phosphate had better buffering effect for the pH change caused by the phosphoric acid formed from the reaction of water and PPi during reaction process (Fig. 1). The yield of the enzymatic reduction increased slightly from 25 to 35 °C.

The K_m and catalytic efficiencies (k_{cat}/K_m) of *Segniliparus* CAR toward **5a** were 404 ± 37 μM and 0.32 μM^{-1} min^{-1}, respectively. They fell in the range of those for *Nocardia* CAR [23], and *M. marinum* CAR (Supplementary Table T2) [13].

3.2. Substrate specificity

The substrate specificity of *Segniliparus* CAR was examined with the purified enzyme. The results in Table 1 showed that a series of aromatic and aliphatic carboxylic acids were reduced to their corresponding aldehydes. For the aliphatic acids, the reduction of nonanoic acid (**2a**) resulted in higher yields than those of hexanoic acid (**1a**) and myristic acid (**3a**). *Segniliparus* CAR also showed high activity toward aromatic carboxylic acids. For examples, **5a**, **23a**, 3-phenylpropanoic acid (**26a**), and 4-phenylbutyric acid (**30a**) were reduced completely. *Ortho*-substituted benzoic acids [hydroxyl (**6a**), methoxyl (**9a**), fluoro (**12a**), chloro (**14a**), and bromo (**16a**)] were the poorer substrates than their corresponding *meta*-substituted ones (**7a**, **10a**, **13a**, **15a**, **17a**). This enzyme was less active toward 2-phenylpropionic acid [**24a**, (*S*)-**24a**] and 3-phenylbutyric acid (**27a**) than **23a** and **26a**, respectively. These results suggested that the activity of *Segniliparus* CAR might be strongly influenced by the steric demand surrounding carboxylic groups, which was consistent with the results for *P. furiosus* (whole-cell) [8] and *Nocardia* CAR [23]. *Segniliparus* CAR showed good chemoselectivity for the reduction of some carboxylic acids containing C=C or C=O double bonds, such as linoleic acid (**4a**), *trans*-cinnamic acid (**28a**), **29a**, and 3-benzoylpropionic acid (**31a**). These acids were reduced, with C=C or C=O double bonds remaining unaffected. However, this enzyme was less active toward **28a** and 3-benzoylacrylic acid (**32a**) than their saturated counterparts (**26a** and **31a**). Interestingly, *Segniliparus* CAR could effectively catalyze the reduction of **8a** and **19a**, toward which *Nocardia* CAR exhibited no or little activity [23]. The complete conversion of both rac-**24a** and (*S*)-**24a** (5 mM) under identical conditions suggested that *Segniliparus* CAR had low enantioselectivity toward racemic **24a**. For ibuprofen [rac-**33a** and (*S*)-**33a**], the conversion of rac-**33a** (14% yield of rac-**33b**) was higher than (*S*)-**33a** [3% yield of (*S*)-**33b**], suggesting that *Segniliparus* CAR might be more active toward the (*R*)-enantiomer than (*S*)-enantiomer, but the enantioselectivity was also low. Similarly, rac-**24a**, rac-**27a** and rac-**33a** were also almost completely reduced by *M. marinum* CAR [13] in our experiments (data not shown). However, by comparing V_{max}/K_m ratios of *Nocardia* CAR toward (*R*)-**33a** and (*S*)-**33a**, the enantiomeric ratio was calculated to be 40.4 for the (*R*)-isomer over the (*S*)-isomer [23]. The conversion of rac-**33a** catalyzed by *Nocardia* sp. indicated that *Nocardia* CAR was R enantioselective, giving the chiral alcohol product with an enantiomeric excess of 61.2% [7].

Table 1
Substrate specificity of *Segniliparus* CAR.

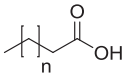
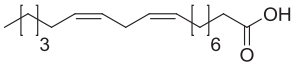
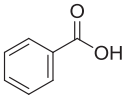
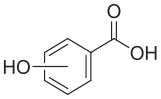
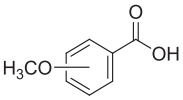
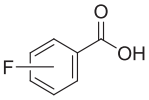
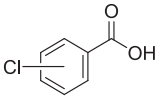
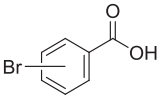
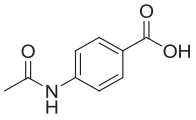
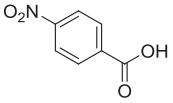
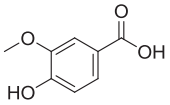
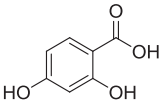
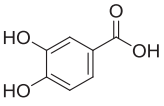
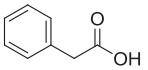
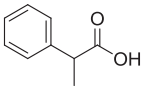
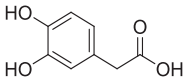
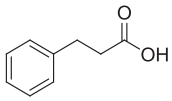
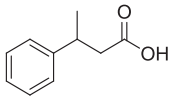
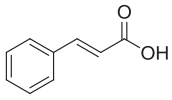
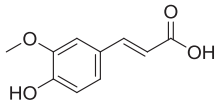
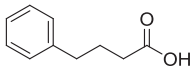
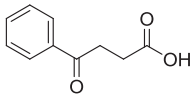
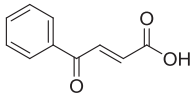
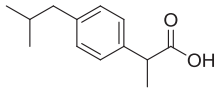
Substrate		Yield (%) of aldehyde
	**1a** (*n* = 3) **2a** (*n* = 6) **3a** (*n* = 11)	79 100 65
	**4a**[a]	6
	**5a**	100
	**6a** (*ortho*)	17
	7a (*meta*)	100
	8a (*para*)	100
	**9a** (*ortho*)	2
	10a (*meta*)	93
	11a (*para*)	100
	**12a** (*ortho*)	82
	13a (*meta*)	86
	**14a** (*ortho*)	18
	15a (*meta*)	41
	**16a** (*ortho*)	2
	17a (*meta*)	70
	**18a**[b]	18
	**19a**[c]	100
	**20a**	93
	**21a**[b]	62
	**22a**[b]	52
	**23a**	100
	**24a** (*R*/*S*)	32; 100[d]
	24a (*S*)	55; 100[d]

Table 1 (*Continued*)

Substrate		Yield (%) of aldehyde
	**25a**[ab]	37
	**26a**	100
	**27a**	94
	**28a**	51
	**29a**[b]	52
	**30a**	100
	**31a**	95
	**32a**	0
	**33a** (*R*/*S*)	14
	33a (*S*)	3

[a] The reaction was performed in sodium phosphate buffer (100 mM, pH 7.5).
[b] Silylation was performed before the GC analysis.
[c] The substrate was taken from 500 mM stock solution in DMSO.
[d] Substrate concentration (5 mM).

3.3. Whole cell catalysis

In order to explore the application potential of *Segniliparus* CAR as a biocatalyst for the reduction of carboxylic acids, the *Segniliparus* CAR and *Nocardia* PPTase genes were co-expressed in *E. coli* BL21(DE3). Without externally adding the cofactors (ATP and NADPH), the recombinant whole cells catalyzed the reduction of **23a** with 48% yield, which was much higher than that using the *E. coli* cells expressing only the *Segniliparus* CAR gene (Table 2) [24,29]. The optimum pH for the whole-cell biotransformation was

Table 2
Reduction of **23a** catalyzed by *E. coli* BL21(DE3)/pETDuet-1-PPTase-CAR and *E. coli* BL21(DE3)/pET32a(+)-CAR.[a]

Conditions	Yield (%) of **23c**
E. coli BL21(DE3)/pET32a(+)-CAR + glucose	14
E. coli BL21(DE3)/pETDuet-1-PPTase-CAR	14
E. coli BL21(DE3)/pETDuet-1-PPTase-CAR + glucose	48

[a] Reaction conditions: sodium phosphate buffer (1 mL, 100 mM, pH 7.5) contained glucose (22.2 mM), *E. coli* BL21(DE3)/pET32a(+)-CAR or *E. coli* BL21(DE3)/pETDuet-1-PPTase-CAR (wet cells, 100 mg), **23a** (10 mM); 16 h, 30 °C, 200 rpm.

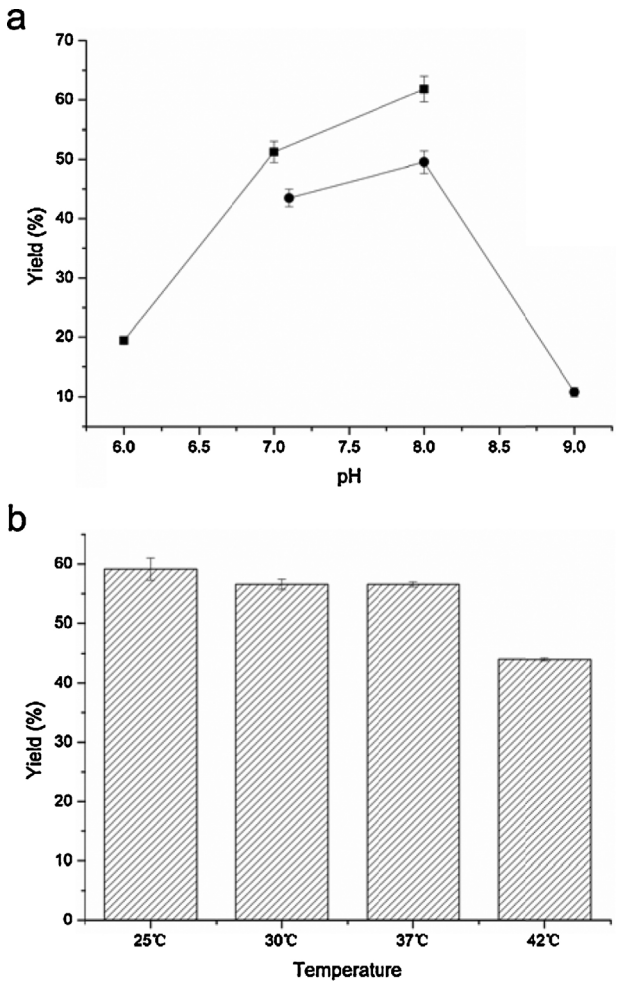


Fig. 2. Effects of pH (a) and temperature (b) on the reduction of **23a** to **23c** by the whole cells. (a) Reaction conditions: different buffers [1 mL, *square* 100 mM sodium phosphate buffer (pH 6.0–8.0), *round* 100 mM Tris–HCl buffer (pH 7.1–9.0)] contained glucose (22.2 mM), *E. coli* BL21(DE3)/pETDuet-1-PPTase-CAR (wet cells, 50 mg), **23a** (5 mM); 16 h, 30 °C, 200 rpm. (b) The reaction at the optimum pH was carried out at different temperatures (25 °C, 30 °C, 37 °C and 42 °C).

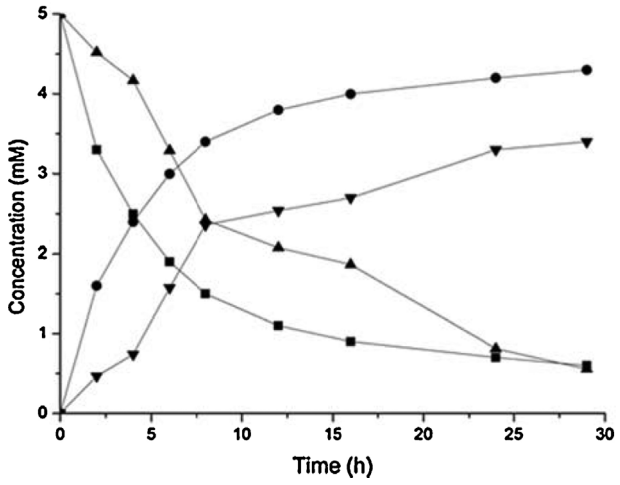


Fig. 3. Time courses for the reductions of **20a** (*square*) to **20c** (*circle*) and **25a** (*uptriangle*) to **25c** (*downtriangle*) by the whole cells.

Table 3
Reduction of **29a** by various amount of whole cells.[a]

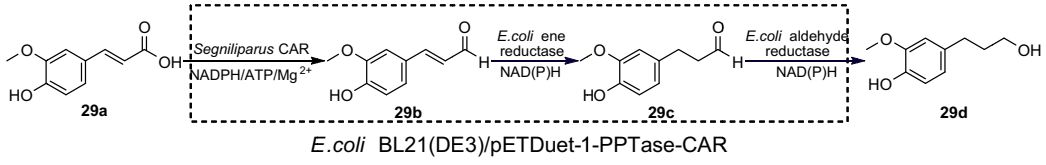
Conditions	Yield (%) of **29b** and **29d**	Ratio of **29d**/**29b**
100 mg cells + 5 mM **29a**	>90	12/88
100 mg cells + 2.5 mM **29a**	>90	46/54
200 mg cells + 2.5 mM **29a**	>90	76/24

[a] Reaction conditions: sodium phosphate buffer (1 mL, 100 mM, pH 8) contained glucose (22.2 mM), *E. coli* BL21(DE3)/pETDuet-1-PPTase-CAR (wet cells), and **29a**; 16 h, 30 °C, 200 rpm.

at pH 8 (Fig. 2), while the reaction temperature did not exert significant effect on the reaction rate in the range of 25–37 °C, although the yield decreased at 42 °C. The reductions of **20a** and **25a** were carried out at pH 8 and 30 °C. As shown in Fig. 3, the conversions of **20a** and **25a** gradually increased over time, reaching up to 87% and 69% at 29 h. **20c** (18.6 mg L^{-1} h^{-1} productivity) and **25c** (11.3 mg L^{-1} h^{-1} productivity) were isolated in 69% and 42% yields, respectively. The lower isolated yield of **25c** was due to its higher solubility in aqueous solution and the instability of **25a** and protocatechualdehyde (**25b**) under alkaline conditions (pH 8). The *E. coli* cells co-expressing *Nocardia* CAR and PPTase catalyzed the reduction of **20a** to give vanillin (**20b**) as the major product together with **20c** [24]. Recently, Napora-Wijata et al. [29] reported the synthesis of **25c** through the reduction of **25a** using whole *E. coli* BL21 (DE3) cells co-expressing *Nocardia* CAR and a PPTase from *E. coli*, only 19% yield and 2 mg L^{-1} h^{-1} productivity were achieved after 144 h. The endogenous aldehyde reductases were proposed to be responsible for the further reduction of the aldehyde product [24,29].

In contrast to **20a** and **25a**, coniferyl aldehydes (**29b**) and **29d** were produced when **29a** was reduced by the whole cells co-expressing the *Segniliparus* CAR and *Nocardia* PPTase genes. This is different from the reduction catalyzed by the recombinant *E. coli* cells harboring the *Nocardia* CAR and PPTase, in which **29b** and coniferyl alcohol were obtained [24]. The purified *Segniliparus* CAR did not catalyze the reduction of C=C bond of **29a**, so we speculated that **29a** was first reduced to **29b** by the recombinant CAR and **29b** was further reduced to **29d** by endogenous ene reductase and aldehyde reductase (Scheme 1). This is consistent with the observation that the ratio of **29d**/**29b** increased when more whole cell biocatalyst was used (Table 3).

Carboxylic acid reductases require Mg^{2+}, ATP, and NADPH as cofactors, and the use of whole cells could avoid the introduction of external cofactor regeneration system into the reaction. However, the diffusion of the substrate into the cell and the insufficient intracellular cofactors may decrease the efficiency of the reaction. Therefore, high cell loading or low conversion until now has been observed for the reduction of carboxylic acids with whole cell biocatalysts [24,29]. Although the studies on ATP/NADPH regeneration



Scheme 1. Proposed process for the reduction of **29a** by the whole cells.

have been carried out [30,31], and the cofactor NADPH regeneration by GDH has been brought into the reduction of carboxylic acid [24], the challenge in the recycling of these cofactors has not been effectively addressed. Our attempt to bring ATP regeneration into the reaction with crude enzymes was also unsuccessful.

4. Conclusion

A gene encoding the thioester reductase domain-containing protein from *S. rotundus* DSM 44985 was successfully overexpressed in *E. coli*. The purified recombinant His-CAR exhibited a broad substrate spectrum and was active toward both aliphatic and aromatic carboxylic acids. Other functional groups such as keto groups and C=C double bonds remained unaffected. The *Segniliparus* CAR and *Nocardia* PPTase genes were co-expressed in *E. coli* BL21(DE3), and the recombinant whole cells convert vanillic acid (**20a**) and 3,4-dihydroxyphenylacetic acid (**25a**) to give vanillyl alcohol (**20c**) and 3-hydroxytyrosol (**25c**), respectively, with the concurrent function of the endogenous aldehyde reductases of *E. coli*. As such, *Segniliparus* CAR may serve as a useful biocatalyst for the reduction of carboxylic acids and find applications in the synthesis of valuable compounds such as vanillyl alcohol (**20c**) and 3-hydroxytyrosol (**25c**), and/or be integrated into the metabolic pathways for producing carboxylic acid-derived biochemicals.

Acknowledgements

This work was financially supported by National Basic Research Program of China (973 Program, No. 2011CB710800) and the Chinese Academy of Sciences (KSZD-EW-Z-015).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.01.014.

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