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A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol

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ABSTRACT

The reduction of acyl-CoA or acyl-ACP to fatty alcohol occurs via a fatty aldehyde intermediate. In prokaryotes this reaction is thought to be performed by separate enzymes for each reduction step while in eukaryotes these reactions are performed by a single enzyme without the release of the intermediate fatty aldehyde. However, here we report that a purified fatty acyl reductase from *Marinobacter aquaeolei* VT8, evolutionarily related to the fatty acyl reductases in eukaryotes, catalysed both reduction steps. Thus, there are at least two pathways existing among prokaryotes for the reduction of activated acyl substrates to fatty alcohol. The *Marinobacter* fatty acyl reductase studied has a wide substrate range in comparison to what can be found among enzymes so far studied in eukaryotes.

1. Introduction

Long-chain primary fatty alcohols are commonly found in their free form or as a component of wax esters in plants, insects and mammals. Often these compounds are building blocks of boundaries as epicuticular wax layers towards the outside environment. Fatty alcohols are synthesised via reduction of acyl-CoA or acyl-ACP and can be further esterified to acyl-CoA resulting in wax esters [1–4]. Wax esters are neutral lipids that are also produced in a few bacterial genera [5–7]. In bacteria, wax esters are most likely serving as energy reserves and are often found in marine environments and associated with species that can degrade crude oil or in marine sediments [8–10]. It can be anticipated that medium and long-chain hydrocarbons from oil spills and decaying organic matter in this way can be utilized for accumulating dense energy reserves.

The bacterial pathway leading to wax ester production has mainly been studied in the genus *Acinetobacter* which synthesise and store wax esters under nitrogen-limiting conditions [11–14]. The medium and long-chain fatty alcohols used for the biosynthesis of wax esters in prokaryotes are believed to be produced via a two-step reduction of either acyl-CoA or acyl-ACP to the corresponding fatty alcohol via an intermediate fatty aldehyde [15,16]. This is contrasting to eukaryotes where a single enzyme can perform both reduction steps without releasing the intermediate fatty aldehyde [4]. A study of mutants of *Acinetobacter calcoaceticus* deficient in wax ester biosynthesis revealed a mutant impaired in the first reduction step leading to fatty aldehyde [14]. Expression of the corresponding gene, *acr1*, in *Escherichia coli* and assays of extracts resulted in aldehyde production. Although production of alcohol could also be observed this was attributed to endogenous activity of *E. coli* that could further reduce the aldehyde to fatty alcohol. A mutation in the second reduction step from aldehyde to alcohol was not identified.

In a recent paper a putative gene sequence was identified in *Marinobacter aquaeolei* VT8 which corresponding protein product was shown to reduce added aldehyde substrates to fatty alcohol and this enzyme was termed FALDR (Fatty Aldehyde Reductase) [17].

We have expressed and purified hypothetical protein Maqu_2220 of *M. aquaeolei* VT8, which is identical with the *M. aquaeolei* VT8 FALDR enzyme. In this report we show that the *Marinobacter* enzyme can, in addition to reduce fatty aldehydes, also reduce acyl-CoAs and acyl-ACP to fatty alcohols and thus perform both reduction steps, thereby establishing that also in prokaryotes both these reductions can be performed in an NADPH-dependent manner by a single enzyme. Thus, contrary to what previously has been anticipated in the literature, there are at least two pathways available among prokaryotes for the reduction of activated acyl chains to fatty alcohols. The *M. aquaeolei* VT8 fatty acyl reductase

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was furthermore shown to be active with acyl-CoA as well as acyl-ACP and with substrates of different chain lengths and modifications.

2. Materials and methods

2.1. Vector construction

DNA with sequence corresponding to GenBank accession YP_959486 (hypothetical protein Maqu_2220 [*M. aquaeolei* VT8]) was ordered synthetically (Eurofins). The synthetic gene sequence was optimised for *E. coli* expression at ordering. The synthetic gene was amplified in a two-step procedure using Phusion™ High-Fidelity PCR (Finnzymes) essentially according to the manufacturer's instructions. Primers for amplification were designed to introduce *att*B-sites for Gateway® cloning as well as a sequence which upon fusion with the gene sequence introduced a TEV protease cleavage site into a resulting expressed protein (Appendix A). The purified PCR fragment was introduced into pDONR™221 using BP Clonase™ II (Invitrogen) resulting in plasmid, pEntry-MarFAR. After purification and sequence verification the modified gene was further introduced into pDEST-HisMBP [18] using LR Clonase™ II Plus (Invitrogen) resulting in plasmid pHisMBPMarFAR.

2.2. Heterologous protein expression and purification

Plasmid pHisMBPMarFAR was transformed to *E. coli* strain Rosetta (DE3) (Novagen) for heterologous protein expression.

A single colony was used to inoculate 40 ml of Luria-Bertani broth (LB) supplemented with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol. The culture was incubated overnight at 37 °C with agitation. A volume of the overnight culture was added to 800 ml antibiotic supplemented LB media to a final OD 600 of 0.1 and then incubated at 37 °C with 240 rpm until OD 600 was 0.5–0.8 (90–100 min). The culture was transferred to room temperature for 30 min and then protein expression was induced by the addition of Isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The induced culture was allowed to grow for another 4–6 h at room temperature under agitation, 240 rpm. Bacteria were pelleted by centrifugation at 5000×*g* for 20 min at 4 °C. The bacterial pellet was flash frozen in liquid nitrogen and stored at −80 °C for subsequent processing.

The bacterial pellet was resuspended in 16 ml ice cold 100 mM phosphate buffer, pH 7.0 containing 40 mM imidazole, 10% (v/v) glycerol, 1× Complete Protease Inhibitor (Roche Applied Bioscience) and 16 μl Lysonase (Novagen). Ice cold 0.1 mm glass beads were added to the bacterial suspension at a ratio of 1:1 (w/v). The suspension was homogenized using a FastPrep™-24 (MP Biomedicals) at 4 m/s, using 3 pulses of 30 seconds with 2 min rest at 4 °C between pulses. The cell lysate was clarified by centrifugation at 4000×*g* for 10 min at 4 °C. MgCl2, ATP and KCl were added to 5 mM, 2 mM and 150 mM respectively. Subsequently the lysate was mixed and incubated on ice for 10 min. The lysate was then further clarified by centrifugation at 10 000×*g* for 10 min at 4 °C.

The supernatant was loaded on a pre-equilibrated 1 ml HisTrap™ HP column (GE Healthcare) with a flow rate of 1 ml/min using a BioLogic LP liquid chromatography system (Bio-Rad). The column was then washed with 100 mM phosphate buffer, pH 7.0 containing 40 mM imidazole and 10% (v/v) glycerol at a flow rate of 1 ml/min. Bound protein was eluted using a 20 ml linear gradient of 40–500 mM imidazole in 100 mM phosphate buffer, pH 7.0 with 10% (v/v) glycerol at a flow rate of 1 ml/min. Eluted proteins were collected in 0.5 ml fractions and subsequently aliquoted, flash frozen in liquid nitrogen and stored at −80 °C until further analysis and enzyme assays. Protein concentration of individual fractions was determined using BCA Protein Assay (Pierce). Protein integrity of individual fractions was determined using polyacrylamide gel electrophoresis (PAGE).

2.3. Substrate synthesis

[^{14}C]-labelled fatty acyl-chains were either obtained from commercial sources or synthesised.

[1-^{14}C]ricinoleic (12-hydroxy-octadec-9-enoic) acid was synthesized biochemically from [1-^{14}C]oleic acid by castor bean microsomes according to Bafor et al. [19].

[1-^{14}C]-labelled straight-chain fatty acids were prepared starting with the next lower fatty alcohols, which were brominated by refluxing with 48% HBr in the presence of tetrabutylammonium bromide as phase transfer catalyst. The bromides were dissolved in dry dimethyl sulfoxide and treated with [^{14}C]-sodium cyanide at 65 °C for 22 h. Following alkaline hydrolysis and purification by reversed-phase HPLC, materials of >98% purity were obtained. The specific radioactivity as determined by mass spectrometry and liquid scintillation counting was 389 kBq/μmol.

Acyl-CoA subtrates were synthesised essentially according Sànchez et al. [20]. [^{14}C]palmitoyl-ACP was synthesised enzymatically from free acid and ACP using *E. coli* acyl-ACP synthetase (provided by Prof. John Ohlrogge, East Lansing, USA) according to Kuo and Ohlrogge [21].

2.4. Enzyme assay

Assays were performed in 50 μl of 100 mM phosphate buffer, pH 7.0 containing 10 mM NADPH, 50 μM [^{14}C]fatty acyl-CoA or –ACP and 3 mg/ml BSA using 0.45 μg of purified protein. Reactions were incubated at 30 °C for 30 min and then stopped by the addition of 10 μl glacial acetic acid (HAc) and immediately extracted with thorough vortexing using 375 μl methanol:chloroform 1:1 (v/v), 125 μl chloroform and 250 μl H_2O. After centrifugation at 6000×*g* for 2 min the chloroform phase was transferred to a fresh vial.

Extracted lipids were separated on TLC Silica gel 60 (Merck) using hexane:diethyl ether:HAc at 55:45:0.5 (v/v/v/) as a mobile phase. Radioactive emission was measured for up to 15 hours using electronic radiography (Instant Imager, Canberra Packard) with [^{14}C]oleyl alcohol (kindly provided by Bayer CropScience) of known specific activity applied on the TLC plate as a standard.

The identity of enzyme reaction products were determined by GC–MS (Appendix C).

2.5. Sequence analysis

Bacterial protein sequences homologous to *M. aquaeolei* VT8 accession YP_959486 were collected by conducting BLASTP against all assembled RefSeq genomes in the Microbes section at NCBI.

A phylogenetic tree of selected fatty acyl-CoA reductase amino acid sequences was assembled by using ClustalW-alignment and then the Neighbour-Joining algorithm with Bootstrapping of CLC DNA Workbench (CLC bio).

3. Results and discussion

3.1. Expression and purification of hypothetical protein Maqu_2220

Hypothetical protein Maqu_2220 of *M. aquaeolei* VT8 has the same amino acid sequence as NCBI reference sequence YP_959486. The corresponding ORF was produced synthetically and inserted into an *E. coli* expression system using Gateway™ cloning. In the chosen expression system Maqu_2220 was expressed as a fusion protein with the maltose binding protein (MBP) which has