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Short communication

Asymmetric synthesis of a fluoxetine precursor with an artificial fusion protein of a ketoreductase and a formate dehydrogenase

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ABSTRACT

Herein we describe the kinetic characterization of a fusion protein from the 3-ketoacyl-[acyl-carrier-protein]-reductase (KR) from *Synechococcus* PCC 7942 and a mutant formate dehydrogenase from *Mycobacterium vaccae* N10 (MycFDH). Upon purification, a specific proteolytic cleavage of the MycFDH was observed. The cleavage site was elucidated, which is ubiquitously spread among prokaryotic FDHs. After depletion of the cleavage site the correct, full length fusion protein was obtained. In asymmetric reductions of ethylbenzoyl acetate (EBA) this fusion protein performed equal or even better than the free enzymes, yielding up to 39% more of the fluoxetine precursor ethyl-(*S*)-3-hydroxy-3-phenylpropanoate ((*S*)-HPPE). The rate acceleration is due to an improved $K_{m,EBA}$ of the KR subunit.

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1. Introduction

Contrary to many multifunctional enzyme complexes, which are post-translationally linked or adjoined, multifunctional fusion proteins are obtained by the connection of two or more separately encoded proteins into one new gene. The new protein then features multiple enzymatic abilities, which originate from the fused subunits [1–3]. A common example for a natural fusion protein is the fatty acid synthase I (FASI) found in animals and fungi. Whereas the equivalent bacterial FASII protein is a multi-enzyme complex, the FASI comprises all the respective catalytic sites within subdomains in one polypeptide chain [4]. The eukaryotic FASI is therefore considered an evolutionary descendant of the bacterial FASII [5].

To enforce biotechnological processes, this principle is applied to enzymes which are sequentially employed in biosynthesis reactions. The general advantage of multifunctional fusion proteins is the inherent co-localization of the catalytic subunits. Moreover, the thereby achieved locally enhanced substrate concentrations are assumed to be an advantage for the catalytic activity of many fusion proteins [1,6]. Regarding industrial applications, fusion proteins offer the option to combine enzyme properties as needed for the specific application. The multifunctional protein then requires only one, instead of several separate production steps. Fusion proteins have so far been the subject of several studies [1–3,7–10]. One example is the fusion of oxidoreductases, a large group of enzymes used for site- and stereo-selective oxidation or reduction. Some oxidoreductases are frequently studied for the application in asymmetric reductions [11]. The 3-ketoacyl-[acyl-carrier-protein]-reductase [EC 1.1.1.100] (KR) from *Synechoccocus* PCC 7942 offers high enantiomeric excess (ee) in the reduction of pro-chiral ketones such as ethylbenzoyl acetate (EBA) [12]. The corresponding alcohol ethyl-(*S*)-3-hydroxy-3-phenylpropanoate ((*S*)-HPPE) (Fig. 1) is a precursor in the production of fluoxetine, the active component of the antidepressant Prozac [13,14]. Oxidoreductases use cofactors, mostly NAD(P)H, for catalysis. As NAD(P)H is highly expensive, an efficient cofactor regeneration is necessary to achieve a profitable process. For industrial purposes oxidoreductases such as the KR are combined to two-enzyme-systems with cofactor regenerating enzymes [11]. Formate dehydrogenases (FDHs) [EC 1.2.1.2] are still considered a gold standard for cofactor regeneration because of many favorable properties such as the irreversibility of the reaction due to CO_2-formation.

FDHs are ubiquitously found in plants, yeast, bacteria and fungi, where they catalyze the oxidation of formate to CO_2 coupled to the reduction of $NAD(P)^+$ [15,16]. In industrial applications FDHs are frequently employed for cofactor regeneration [17]. Therefore, many attempts were made to improve the catalytic properties of

Abbreviations: EBA, ethylbenzoyl acetate; FDH, formate dehydrogenase; (*S*)-HPPE, ethyl-(*S*)-3-hydroxy-3-phenylpropanoate; KR, 3-ketoacyl-[acyl-carrier-protein]-reductase.

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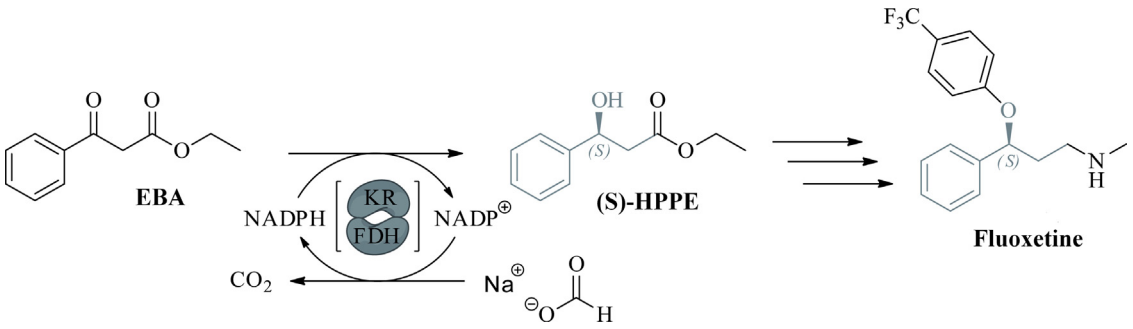

Fig. 1. Scheme of the asymmetric reduction of ethylbenzoyl acetate (EBA). Scheme of the asymmetric reduction of ethylbenzoyl acetate (EBA) to ethyl-(*S*)-3-hydroxy-3-phenylpropanoate ((*S*)-HPPE) by the ketoreductase (KR) subunit of the fusion protein. (*S*)-HPPE is subsequently incorporated in the synthesis of fluoxetine [14]. The structural part of the product originating from the chiral alcohol is highlighted in gray. The regeneration of NADPH is catalyzed by the oxidation of sodium formate to CO_2 by the formate dehydrogenase (FDH) subunit of the fusion protein.

these enzymes using protein engineering. As FDHs originate from a catabolic pathway, they favor NAD^+ over $NADP^+$ [18]. One aim was therefore to engineer enzymes with a switched cofactor preference. Additionally, FDHs were modified regarding thermal and chemical stability, especially focusing on highly reactive cysteines [15,16,19–21].

First attempts to create a multifunctional fusion protein of KR and FDH for parallel and efficient asymmetric reduction and cofactor regeneration were described by Hölsch and Weuster-Botz [9]. For cofactor regeneration a mutant FDH from *Mycobacterium vaccae* N10 (MycFDH) with enhanced activity toward $NADP^+$ was chosen and combined with the KR from *Synechococcus* PCC 7942. As the MycFDH is a dimeric enzyme [18] and the native KR exists as an homotetramer [12], the fusion of both enzyme moieties led to the formation of a multimeric protein network [9]. In whole cell biotransformations this fusion protein achieved higher yields in asymmetric reductions compared to the expression of the separate enzymes. So far, the enzymatic characterization was restricted to whole cell biotransformations or crude extracts. For a better understanding of the advantages of artificial multifunctional fusion proteins in coupled enzyme catalysis, a thorough analysis of the purified enzyme and the fused subunits is of interest.

This report covers our studies on a fusion protein consisting of MycFDH and KR (Fig. 1). It summarizes the enzymatic characterization of both functional subunits and analyses the properties of the isolated multifunctional enzyme in coupled asymmetric synthesis of the fluoxetine precursor (*S*)-HPPE in comparison to the separate enzymes.

2. Materials and methods

2.1. Chemicals

EBA (>93.0%) and ethyl-(*R*)-3-hydroxy-3-phenylpropanoate ((*R*)-HPPE) were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (Schnelldorf, Germany), respectively. Oligonucleotides were synthesized by Eurofins Genomics (Ebersberg, Germany). All other chemicals were purchased in analytical grade from Carl Roth (Karlsruhe, Germany) and were used without further purification.

2.2. Cloning and site-directed mutagenesis

Standard methods were used for cloning and site-directed mutagenesis procedures, which are described in the Supplemental material.

2.3. Protein purification

All enzymes were expressed as described by Hölsch and Weuster-Botz [9]. Purification was conducted using immobilized metal affinity chromatography (IMAC) with 1 mL HisTrap FF crude columns (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The enzymes were then dialyzed against 0.1 M sodium phosphate buffer (pH 7.0).Protein expression and progress in protein purification were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie-staining. A bicinchoninic acid assay (Pierce, Rockford, USA) was used to determine the protein concentration according to the manufacturer's instructions.

2.4. Enzyme activity assays

FDH activity was measured photometrically at 340 nm with protein concentrations of ≤20 μg/mL using a 0.1 M sodium phosphate buffer (pH 7.0). Unless stated otherwise, all experiments were performed at 22 °C. As FDHs catalyze bi-substrate reactions, either formate or $NADP^+$ was added in excess. Enzyme activities were calculated using a molar extinction coefficient $\varepsilon_{340\,nm} = 6300\,L\,mol^{-1}\,cm^{-1}$.

KR activity and the ee of (*S*)-HPPE were determined by chiral gas chromatography using a Lipodex-E column (25 m × 0.25 mm i.d., Macherey-Nagel, Düren, Germany) and 7.2 mM 2-heptanone as internal standard. The injector and detector temperatures were adjusted to 250 °C and 220 °C, respectively. The temperature gradient was programmed from 45 °C to 110 °C at 5 °C min^{-1} (30 min isothermal) and from 110 °C to 200 °C at 15 °C min^{-1} (10 min isothermal). Retention times at a flow-rate of 4 mL min^{-1} helium were: 2-heptanone, 4.0 min; EBA, 30.8 min; (*R*)-HPPE, 39.2 min; (*S*)-HPPE, 40.0 min.

Coupled enzyme reactions were conducted using 30 μg/mL of the fusion protein or the equivalent with a 2:1 mass ratio (MycFDH(M3):KR) of the separated enzymes. 4 mM EBA were reduced in the presence of different $NADP^+$ concentrations (0.1–2.5 mM) and 500 mM formate at 30 °C in a total volume of 1.4 mL.

All calculations were done using SigmaPlot 8.0 (SPSS, Surrey, UK) or Matlab 2013b (MathWorks, Natick, MA, USA).

2.5. Mass spectrometry

To identify and analyze the proteins in the observed bands on SDS-PAGE, spots were excised and digested with trypsin following the protocol of Schäfer et al. [22]. The spectra were recorded and identified with an Ultraflex I matrix-assisted laser desorption–ionization time-of-flight/time-of-flight mass

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