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Coupling carboxylic acid reductase to inorganic pyrophosphatase enhances cell-free *in vitro* aldehyde biosynthesis

Aditya M. Kunjapur [a,b,1], Bernardo Cervantes [c], Kristala L.J. Prather [a,b,c,*]

[a] Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[b] Synthetic Biology Engineering Research Center (SynBERC), Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[c] Microbiology Graduate Program, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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ABSTRACT

Carboxylic acid reductases (CARs) have been harnessed in metabolic pathways to produce aldehydes in engineered organisms. However, desired aldehyde products inhibit cell growth and limit product titers currently achievable from fermentative processes. Aldehyde toxicity can be entirely circumvented by performing aldehyde biosynthesis in non-cellular systems. Use of purified CARs for preparative-scale aldehyde synthesis has been limited by *in vitro* turnover of model CARs, such as Car_{Ni} from *Nocardia iowensis*, despite robust conversion of substrates associated with expression in heterologous hosts such as *E. coli* and yeast. In this study, we report that *in vitro* activity of Car_{Ni} is inhibited by formation of the co-product pyrophosphate, and that pairing of an inorganic pyrophosphatase (Ppa_{Ec}) with Car_{Ni} substantially improves the rate and yield of aldehyde biosynthesis. We demonstrate that, in the presence of Ppa_{Ec}, Michaelis–Menten kinetic models based on initial rate measurements accurately predict Car_{Ni} kinetics within an *in vitro* pathway over longer timescales. We rationalize our novel observations for Car_{Ni} by examining previously posed arguments for pyrophosphate hydrolysis made in the context of other adenylate-forming enzymes. Overall, our findings may aid in increasing adoption of CARs for cell-free *in vitro* aldehyde biosynthetic processes.

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1. Introduction

Aldehydes find uses in many industries, including the flavors and fragrances industries. Vanillin and benzaldehyde, which are two aromatic aldehydes that respectively provide vanilla and almond flavors, are the two flavor additives to food products with the largest global annual markets by quantity [1–3]. Other aldehydes with relevance to the flavor industry are aliphatic (fatty) aldehydes such as hexanal, octanal, decanal, and dodecanal, as well as terpenoid aldehydes such as citral and safranal [4–6]. Plant extracts that naturally contain desired aldehydes for flavor applications are often expensive and/or scarce, most notably in the cases of vanillin and safranal [6,7]. However, in the flavor industry and other industries requiring high purity, additives that are produced biologically without the use of harsh chemicals or severe processing conditions are often considered "natural" from a regulatory perspective and may be priced commensurate with their plant-derived counterparts [8]. If markets are sufficiently large, such molecules may represent more attractive targets than commodity chemicals for the development of biotechnological production processes.

Two broad classes of bioprocesses are capable of generating aldehydes from more abundant and affordable natural precursors: (i) whole-cell microbial conversion processes; or, (ii) cell-free conversion processes [9,10]. Advantages of using microbial conversion include the ability to use less expensive inputs and more easily achieve economies of scale through large fermentations. However, cell-based aldehyde synthesis processes are limited by the rapid reduction of aldehydes to alcohols by endogenous reductases [11,12] and by toxicity to the host cell [12–15]. Aldehyde reduction can be minimized by deleting genes encoding endogenous reductases [12]; however, mitigation of toxicity effects remain unsolved. Cell-free or *in vitro* biosynthetic routes could be used to circumvent both the aldehyde stability and toxicity issues, with the minor exception of aldehyde adducts that may form on, and inhibit, aldehyde biosynthetic enzymes.

In recent years, carboxylic acid reductases (CARs) have shown outstanding promise for their use in aldehyde biosynthesis [11,16].

The carboxylic acid reductase from *Nocardia iowensis* (Car_{Ni}) is a model CAR that has been characterized *in vitro*, was found to be active on diverse aldehyde substrates ranging across aromatic and aliphatic families, and has been expressed in *Escherichia coli* and *Saccharomyces cerevisiae* to enable microbial production of aldehydes from carboxylic acid precursors that are supplied exogenously to cultures or produced intrinsically from metabolism [12,17–22]. Many of the carboxylic acid precursors can be found more inexpensively in nature than their corresponding desired aldehyde. Carboxylic acids can also serve as the target for microbial production with minimal toxicity, and subsequently, the carboxylic acid product from fermentation can be separated and converted to aldehyde in a cell-free environment. The first report detailing the biosynthesis of vanillin from glucose harnessed such a two stage approach [23]. Like other soluble enzymes, CARs can also be utilized in the three main types of cell-free systems: they can be expressed in cells that are later lysed to generate cell-extracts (CFX); they can be synthesized *in vitro* using dilute aqueous systems that contain transcription (TX) and translation (TL) machinery for cell-free protein synthesis (CF TX–TL or CFPS) [24]; or alternatively, they can be purified and added to dilute aqueous *in vitro* solutions [9,10].

One primary obstacle to the use of purified CARs for cell-free *in vitro* aldehyde biosynthesis is their low conversion of carboxylic acids in dilute systems [18,25]. In this report, we investigate why CARs are subject to apparently limited turnover *in vitro* using the model CAR from *N. iowensis* (Car_{Ni}). We identify that a known co-product of the reaction, pyrophosphate (PP_i), is inhibitory. Addition of inorganic pyrophosphatase sourced from *E. coli* substantially improves turnover, enabling predictable pathway kinetics using simple Michaelis–Menten models and improving final conversion nearly two-fold under some of the conditions investigated.

2. Materials and methods

2.1. Plasmid construction

E. coli DH10B (Invitrogen, Carlsbad, CA) was used for plasmid cloning transformations and plasmid propagation. PCR amplification was performed using custom oligonucleotides (Sigma-Genosys, St., Louis, MO) (Table 1) and Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA). Restriction enzymes were obtained from New England Biolabs. In order to potentially study the effect of enzyme co-localization in a subsequent study, *C*-terminal peptide tags corresponding to synthetic protein scaffold domains appended to flexible glycine–serine linkers were added to Car_{Ni} and to a heterologous aldo-keto reductase from *Bacillus subtilis* (YtbE_{Bs}) prior to expression and purification. In addition, *N*-terminal hexahistidine (His) tags were added to enable nickel-affinity purification. As previously described, the codon-optimized gene encoding Car_{Ni} was first cloned to generate the pET/His-Car-RBS2-Sfp vector [12,22]. Next, the gene encoding Car_{Ni} was amplified by PCR using two sets of oligonucleotides (first "Car-GBD-f" and "Car-GBD-r1", and next "Car-GBD-f" and "Car-GBD-r2") in order to add a sequence encoding the GBD domain cognate peptide to the open reading frame [26]. This amplicon was then cloned into the same site as the original Car_{Ni} using the restriction enzymes BamHI and NotI. The untagged version of Car_{Ni}, which we purified and assayed previously [22], has very similar activity and is subject to the same limited turnover phenomenon. The gene encoding YtbE_{Bs} was amplified from *Bacillus subtilis* PY79 genomic DNA (gDNA) by PCR and then cloned into the pCDFDuet vector (Novagen, Madison, WI) using the restriction enzymes BamHI and SalI. Next, the gene encoding YtbE_{Bs} was amplified by PCR in order to add a sequence encoding the SH3 domain cognate peptide to the open reading frame [26]. This amplicon was then cloned into the same

site as the original YtbE_{Bs} using the restriction enzymes BamHI and SalI. The gene encoding inorganic pyrophosphatase (Ppa_{Ec}) was amplified from *E. coli* MG1655 gDNA and cloned into the pTEV5 vector using the restriction enzymes NdeI and NotI. *B. subtilis* and *E. coli* gDNA were prepared using the Wizard Genomic DNA purification kit (Promega, Madison, WI).

2.2. Chemicals

Commercial inorganic pyrophosphatase (sourced from *E. coli*) was purchased from New England Biolabs. The following compounds were purchased from Sigma: benzoic acid, benzaldehyde, benzyl alcohol, vanillic acid, vanillin, magnesium chloride, dithiothreitol (DTT), ATP disodium salt hydrate, AMP disodium salt, NADPH tetrasodium salt, NADP^+ sodium salt, and sodium pyrophosphate tetrabasic. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Denville Scientific. Ampicillin sodium salt and streptomycin sulfate were purchased from Affymetrix.

2.3. Enzyme purification

All proteins in this study were overproduced using *E. coli* BL21 Star (DE3) obtained from Invitrogen. All proteins were purified using two-step purification techniques to ensure high purity. SDS-PAGE gels of purified proteins are presented in Fig. S1. Car_{Ni} and YtbE_{Bs} were purified using sequential affinity and anion exchange chromatography. An overnight culture harboring either pET/His-Car-GBDtag-RBS2-Sfp or pCDF/His-YtbE-SH3tag was used as 10% (v/v) inoculum in two liters of LB Broth containing either 100 mg/L ampicillin or 50 mg/L streptomycin, respectively. Cultures were incubated at 30 °C and 250 rpm, and expression was induced using a final concentration of 1 mM IPTG at an OD_{600} of 0.6. Cells were harvested after 20 h using centrifugation and resuspended in Buffer A (100 mM MOPS-NaOH [pH 7.0], 300 mM NaCl, and 10% glycerol). Cells were subsequently lysed using sonication. The supernatant was collected, supplemented with imidazole (5 mM) and batch bound at 4 °C for 2 h to 1 ml of Ni-NTA resin (Qiagen, Germantown, MD). The resin was washed with Buffer A containing 7.5 mM imidazole and subsequently poured into a column. Affinity chromatography was performed using step-wise increasing concentrations of imidazole (20, 40, 60, 100, and 250 mM). Fractions containing purified His-tagged enzyme were pooled and dialyzed overnight at 4 °C into Buffer B (100 mM MOPS-NaOH [pH 7.0], 50 mM NaCl, 1 mM DTT, and 10% glycerol).

For subsequent anion exchange chromatography, dialyzed fractions were loaded onto a 5 × 5 ml HiTrap Q HP anion exchange column (GE Life Sciences, Piscataway, NJ) via a superloop, which were integrated into an ÄKTApurifier with a UNICORN control system v5.20 and a Frac-950 collector (GE Life Sciences). The purification was performed at a flow rate of 1 ml/min at 4 °C. An initial wash of 25 ml was followed by a linear gradient from 50 mM NaCl to 500 mM NaCl for 100 ml elution volume. Fractions of 2 ml were collected and absorbance at 280 nm was used to determine desired fractions. Desired fractions were pooled and dialyzed once again in Buffer B to reduce salt content. Dialyzed enzyme was then flash frozen using liquid nitrogen and stored at −80 °C.

The gene encoding Ppa_{Ec} was inserted into the pTEV5 vector for protein purification, leading to an enzyme product containing a His tag removable by treatment with TEV protease. One liter of cells harboring pTEV5/Ppa was grown at 30 °C and 250 rpm in LB medium containing 100 mg/liter of ampicillin. Expression was induced using a final concentration of 1 mM IPTG at an OD_{600} of 0.6. Cells were harvested after 20 h using centrifugation and resuspended in Buffer A. Cells were subsequently lysed using sonication. The supernatant was collected, supplemented with imidazole (5 mM) and batch bound at 4 °C for 2 h to 1 ml of Ni-