Archives of Biochemistry and Biophysics 616 (2017) 47–58

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Structure and biochemistry of phenylacetaldehyde dehydrogenase from the *Pseudomonas putida* S12 styrene catabolic pathway

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ARTICLE INFO

Article history:
Received 18 June 2016
Received in revised form
3 January 2017
Accepted 23 January 2017
Available online 31 January 2017

Keywords:
Styrene monooxygnease
Phenylacetaldehyde dehydrogenase
Structure
Kinetics

ABSTRACT

Phenylacetaldehyde dehydrogenase catalyzes the NAD^+-dependent oxidation of phenylactealdehyde to phenylacetic acid in the styrene catabolic and detoxification pathway of *Pseudomonas putida* (S12). Here we report the structure and mechanistic properties of the N-terminally histidine-tagged enzyme, NPADH. The 2.83 Å X-ray crystal structure is similar in fold to sheep liver cytosolic aldehyde dehydrogenase (ALDH1), but has unique set of intersubunit interactions and active site tunnel for substrate entrance. In solution, NPADH occurs as 227 kDa homotetramer. It follows a sequential reaction mechanism in which NAD^+ serves as both the leading substrate and homotropic allosteric activator. In the absence of styrene monooxygenase reductase, which regenerates NAD^+ from NADH in the first step of styrene catabolism, NPADH is inhibited by a ternary complex involving NADH, product, and phenylacetaldehyde, substrate. Each oligomerization domain of NPADH contains a six-residue insertion that extends this loop over the substrate entrance tunnel of a neighboring subunit, thereby obstructing the active site of the adjacent subunit. This feature could be an important factor in the homotropic activation and product inhibition mechanisms. Compared to ALDH1, the substrate channel of NPADH is narrower and lined with more aromatic residues, suggesting a means for enhancing substrate specificity.

1. Introduction

Styrene is an essential component in the industrial synthesis of plastics, resins, rubbers, and a myriad other polymers. In 2010, approximately 3.75 billion kilograms of styrene were produced in the United States alone [1]. Despite its prevalent industrial utilization, styrene is a classified mutagen, a suspected carcinogen, and considered to be “immediately dangerous to life or health” by the National Institute for Occupational Safety and Health [2]. Moreover, styrene oxide, the immediate degradation product of styrene in the human liver, is a known carcinogen and considered a greater threat than styrene [3]. Together, styrene and highly reactive styrene oxide have been implicated as reproductive toxicants, neurotoxicants, and/or carcinogens [4]. The harmful properties and the emissions and effluents that inevitably accompany large scale production make the efficient removal of styrene from the environment not just desirable, but imperative to ensure public safety. The commercial value of styrene as a synthetic building block juxtaposed with issues of human and environmental toxicology have spurred structural and mechanistic studies of the enzymes involved in microbial styrene metabolism. In addition to the value of the intact styrene degradation pathway for environmental remediation efforts, its component enzymes offer unique biotechnological potential for the synthesis of chiral oxides, aldehydes, acids, and their derivatives.

Pseudomonas bacteria, are equipped with a genomically-encoded styrene catabolon and represent one of the most versatile organisms engaged in the oxidative metabolism of styrene and

Abbreviations: ALDH, aldehyde dehydrogenase; ALDH1, sheep liver cytosolic aldehyde dehydrogenase; ALDH2, bovine mitochondrial aldehyde dehydrogenase; BME, β-mercaptoethanol; CoA, coenzyme A; P-SEA, protein secondary element assignment; (N)PADH, (N-terminally histidine-tagged)phenylacetaldehyde dehydrogenase; rmsd, root mean square deviation; SMO, styrene monoxygenase; SMOA, styrene monooxygenase reductase component; SMOB, styrene monoxygenase oxygenase component; SOI, styrene oxide isomerase; NADH, nicotinamide adenine dinucleotide, reduced form; NAD^+, nicotinamide adenine dinucleotide, oxidized form; PAL, phenylacetaldehyde; PAA, phenylacetic acid.

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http://dx.doi.org/10.1016/j.abb.2017.01.011
0003-9861/

related aromatic and aliphatic hydrocarbons [5,6]. In our work we have focused on enzymes from the styrene metabolic pathway of *P. putida* (S12), an unusually solvent tolerant strain. At the entry point of the styrene pathway, the NADH and FAD–dependent two-component styrene monooxygenase (SMO) transfers an atom from molecular oxygen to the vinyl side chain of styrene to synthesize styrene oxide [7]. This is followed by the transformation of styrene oxide to phenylacetaldehyde (PAL) by the membrane protein, styrene oxide isomerase (SOI) and the NAD^+-dependent oxidation of PAL to phenylacetic acid (PAA) by phenylacetaldehyde dehydrogenase (PADH) [8]. Phenylacetate enters the central metabolic pathways upon its transformation into acetyl-CoA and succinyl-CoA through a series of reactions shared in common with phenylalanine catabolism (Fig. 1) [9].

As part of our continuing work to understand microbial styrene catabolism, we investigated the structure and mechanism of the phenylacetaldehyde dehydrogenase. In general, aldehyde dehydrogenases (ALDHs) are a structurally and mechanistically conserved family of enzymes that catalyze the oxidation of aldehydes to carboxylic acids [10,11] and are distributed ubiquitously across all life kingdoms where they have integral biosynthetic functions ranging from the synthesis of retinoic acid from vitamin A in developmental biology [12] to the production of the lignin, a structural support polymer of plant materials [13]. ALDHs. Pyridine nucleotide-dependent ALDHs, categorized as either Class I, II or III depending on their primary sequence, share similar active site structures but differ in pyridine nucleotide and aldehyde substrate specificity, metal ion-dependence, and quaternary structure assembly [14]. Class-I and II ALDHs assemble as homotetramers and their catalytic activity is dependent on metal ions [14,15]. Class-III enzymes assemble as homodimers and are catalytically insensitive to divalent metal ions [16].

Microbial aldehyde dehydrogenases, which are structurally and mechanistically similar to the human enzymes, are often integrated into pathways for the oxidative transformation of hydrocarbons, alcohols, amines, and amino acids into biosynthetic carbon and energy [17,18]. In the commercial syntheisis of organic acid derivatives these enzymes represent valuable biocatalysts. In the present work we characterize the structure and mechanism of the N-terminally histidine-tagged phenylacetaldehyde dehydrogenase from the styrene catabolic pathway (NPADH) and evaluate the ability of styrene monooxygenase reductase to relieve NADH (product) inhibition and increase the catalytic efficiency of NPADH.

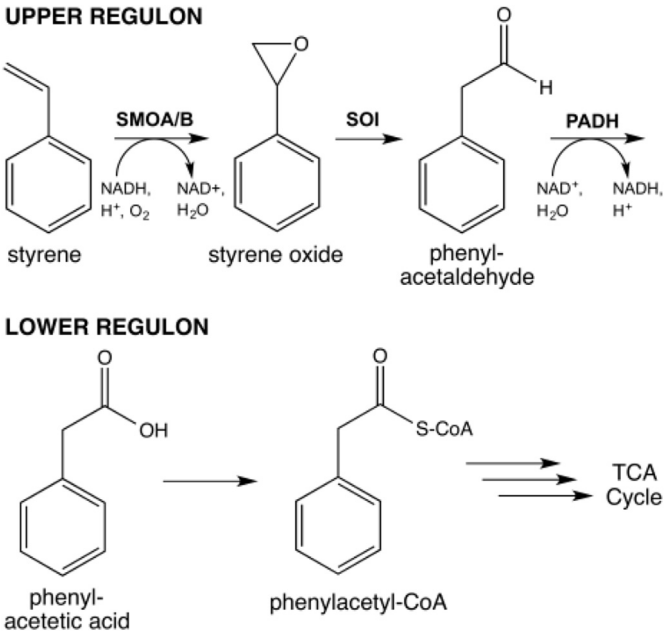

Fig. 1. The styrene catabolic pathway of *P. putida*.

2. Materials and methods

2.1. Cloning and expression system

QIAGEN Genomic-tips (100G) were used to isolate genomic DNA extracted from liquid cultures of *Pseudomonas putida* (S12) per the manufacturers directions. Genomic DNA was concentrated and desalted by isopropanol precipitation. Primers were designed based on the reported DNA sequence of the *StyD* from *Pseudomonas* sp. (Y2) [19]. 5'-phosphorylated primers (forward: 5′-P-CCA-TATGAACAGTTCTCTTTCCGCA-3′ and reverse: 5′-P-AAGTTTT-CATTGTTGTATCTCGCGTAA-3′) were synthesized by Invitrogen, designed for use with *PfuTurbo*® DNA polymerase (Tm = 72 °C) and included a 5′-NdeI restriction site. The *styD* gene was amplified from 100 ng of purified genomic DNA through 30-cycles of PCR in Pfu buffer containing 2.5 mM of each dNTP, and 100 ng of each primer. Next the gene was inserted into a pZeRO-2 (Invitrogen) vector that was blunt end cut with EcoRV and subsequently ligated using T4 DNA ligase. *E.coli* Top10 cells where then transformed with the plasmid by electroporation using a BioRad MicroPulser. Cells containing the recombinant pZeRO-2 plasmid with the *styD* insert were propagated, recovered by using a Qiagen Miniprep plasmid purification kit and sequence verified by Eurofins MWG Operon. The primary sequence of *P. putida* (S12) found to be identical to that of *styD* from *P. florescens* except for amino acid position 63 of the *P. putida* sequence, where an alanine is substituted for glycine.

Before transferring the gene to a pET-28b expression vector, a single Nde1 site located in the cloned gene was eliminated by using the Statagene Quick Change Mutagenesis protocol with the following primer and its complement: 5′-GCAAGGCGTCCA-CATGGGCCCCATGC-3'. DNA sequencing confirmed a T to C substitution and elimination of the internal Nde1 site (CATATG) in *styD* while preserving the histidine codon.

After removal of the Nde1 site, recombinant *styD* was excised from pZeRO-2 by double digestion with Nde1 and Xho1 and recovered after purification by agarose gel electrophoresis. A pET-28b plasmid from Stratagene, double digested with Nde1 and Xho1 and treated with calf intestinal phosphatase to prevent competitive recombination with the excised polycloning site, was combined with recombinant *styD* and ligated by reaction with T4 DNA ligase. The newly generated pET-28 (NPADH) expression vector adds a 20 amino acid His_6-tag and thrombin cut site to the PADH N-terminus. Insertion of *styD* into the expression vector was verified by restriction endonuclease digestion followed by agarose gel electrophoresis. The sequence of the *styD* gene integrated in this expression vector, pET-28 (NPADH), was verified by DNA sequencing. Competent BL21 (DE3) *E. coli* cells were transformed by heat shock, propagated and stored as stocks in 15% glycerol at −80 °C.

2.2. Expression and purification NPADH

NPADH was expressed and purified by using nickel affinity chromatography following protocols similar to those described previously for the preparation of NSMOA [20]. Starter cultures containing 5 mL of sterile LB medium and 30 $\mu g/mL^{-1}$ kanamycin were inoculated with BL21 (DE3) *E. coli* cells containing pET-28(NPADH). After 16 h of aerobic growth at 37 °C with shaking at 250 rpm, starter cultures were expanded to 6 L of the same medium