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Comparison of untagged and his-tagged dihydrodipicolinate synthase from the enteric pathogen *Vibrio cholerae*

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ABSTRACT

Given the emergence of multi drug resistant *Vibrio cholerae* strains, there is an urgent need to characterize new anti-cholera targets. One such target is the enzyme dihydrodipicolinate synthase (DHDPS; EC 4.3.3.7), which catalyzes the first committed step in the diaminopimelate pathway. This pathway is responsible for the production of two key metabolites in bacteria and plants, namely *meso*-2,6-diaminopimelate and *L*-lysine. Here, we report the cloning, expression and purification of untagged and His-tagged recombinant DHDPS from *V. cholerae* (Vc-DHDPS) and provide comparative structural and kinetic analyses. Structural studies employing circular dichroism spectroscopy and analytical ultracentrifugation demonstrate that the recombinant enzymes are folded and exist as dimers in solution. Kinetic analyses of untagged and His-tagged Vc-DHDPS show that the enzymes are functional with specific activities of 75.6 U/mg and 112 U/mg, K_M (pyruvate) of 0.14 mM and 0.15 mM, K_M (*L*-aspartate-4-semialdehyde) of 0.08 mM and 0.09 mM, and k_{cat} of 34 and 46 s^{-1}, respectively. These results demonstrate there are no significant changes in the structure and function of Vc-DHDPS upon the addition of an N-terminal His tag and, hence, the tagged recombinant product is suitable for future studies, including screening for new inhibitors as potential anti-cholera agents. Additionally, a polyclonal antibody raised against untagged Vc-DHDPS is validated for specifically detecting recombinant and native forms of the enzyme.

1. Introduction

Vibrio cholerae is a Gram negative facultative anaerobic comma-shaped bacterium [1]. The organism is the causative agent of cholera and is often present in saline environments, such as estuaries and coastal waters [1]. Cholera is an acute diarrheal disease responsible for significant human morbidity and mortality [1–3]. The disease is associated with pandemic and epidemic outbreaks and is endemic in more than 40 countries, causing 3–5 million infections per annum [1–3].

Reported increases of drug resistance in *V. cholera* strains, including O1 and O139, have highlighted the need to discover new anti-cholera agents and an equally urgent need to characterize novel drug targets [3]. One such target is the enzyme dihydrodipicolinate synthase (DHDPS), which catalyzes the first committed and rate-limiting step in the diaminopimelate (DAP) pathway of bacteria and plants (Fig. 1A) [4–13]. The pathway is associated with *de novo* synthesis of two essential metabolites, namely *meso*-2,6-diaminopimelate (*meso*-DAP) and *L*-lysine [14]. In *Vibrio* spp., *meso*-DAP and *L*-lysine are the fundamental building blocks for the synthesis of Gram negative bacterial cell wall and housekeeping proteins, respectively [14]. DHDPS is the product of an essential bacterial gene, and thus is a promising target for antibiotic development [5,7,9,15]. Accordingly, several studies have recently set out to characterize the structure, function and regulation of this unexploited antibiotic target [5,6,10–13,16–27].

DHDPS is the product of the *dapA* gene [5,9,31]. In *V. cholerae*, the *dapA* gene encodes a 292-amino acid protein with a monomeric molecular mass of 31,348 Da [31]. DHDPS is a class I aldolase that catalyzes the condensation of pyruvate and *L*-aspartate-4-semialdehyde (ASA) to yield the unstable heterocyclic product, 4-hydroxy-2,3,4,5-tetrahydrodipicolinic acid (HTPA) [4,6,8,21,32]. The kinetic properties of several bacterial DHDPS enzymes have been determined, indicating a bi-bi ping-pong kinetic mechanism [5,16,17,27,33,34].

Structurally, DHDPS enzymes have been characterized from numerous bacterial species, including *Agrobacterium tumefaciens* [16], *Bacillus anthracis* [27,35], *Escherichia coli* [30,36], *Mycobacterium tuberculosis* [33], *Streptococcus pneumoniae* [5,26,30] and *Thermotoga maritima* [34], revealing a homotetrameric conformation (Fig. 1B) with each monomer comprising of two distinct domains (Fig. 1C) [8,30,36]. The N-terminal domain folds to form a TIM- or $(\beta/\alpha)_8$-barrel important for catalytic activity; whereas the C-terminal domain consists of three α-helices containing the molecular determinants for tetramerization [8,30,36]. Interestingly, dimeric DHDPS enzymes have recently been

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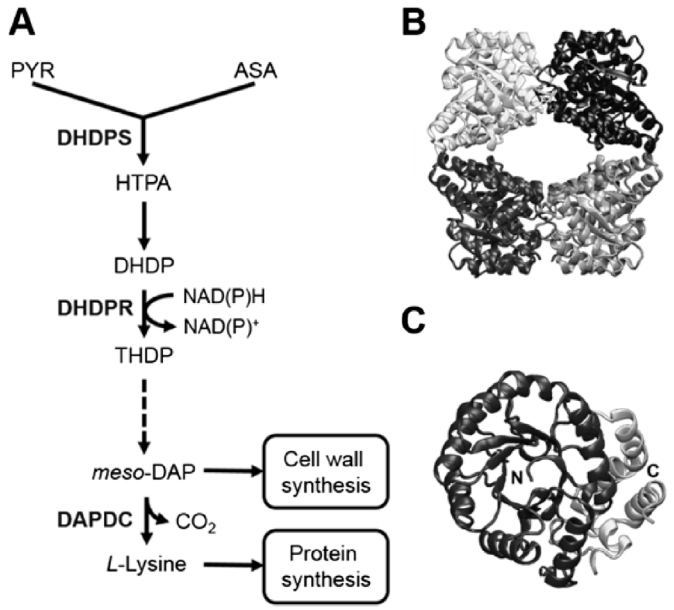

Fig. 1. (A) Diaminopimelate pathway in *V. cholerae*. The first step involves the condensation of pyruvate (PYR) and *L*-aspartate-4-semialdehyde (ASA) by dihydrodipicolinate synthase (DHDPS) to form 4-hydroxy-2,3,4,5-tetrahydrodipicolinic acid (HTPA); which is non-enzymatically dehydrated to form dihydrodipicolinate (DHDP). DHDP is subsequently reduced by dihydrodipicolinate reductase (DHDPR) to form 2,3,4,5-tetrahydrodipicolinate (THDP) [28], which is further processed by several enzymes through the succinylase sub-pathway to yield *meso*-2,6-diaminopimelate (*meso*-DAP). *L*-lysine is then produced by the decarboxylation of *meso*-DAP catalyzed by diaminopimelate decarboxylase (DAPDC) [29]. The products of the pathway, *meso*-DAP and *L*-lysine, are important for cell wall and protein biosynthesis in Gram negative bacteria, respectively; (B) Three-dimensional structure of DHDPS from *Escherichia coli* (PDB ID: 1YXC) [30]; (C) Subunit of *E. coli* DHDPS, highlighting the amino (N) terminal domain in black and the carboxyl (C) terminal domain in grey.

reported from *Pseudomonas aeruginosa* [22], *Staphylococcus aureus* [17], *Legionella pneumophila* [26] and *Shewanella benthica* [37,38].

Introduction of affinity tags, such as a hexa-histidine (His) tag, can assist in purification and crystallization of recombinant proteins [28,39,40]. Thus, this study describes the cloning, expression and purification of untagged and His-tagged *V. cholerae* DHDPS (Vc-DHDPS). We also present a comparative in-solution structural and functional characterization of both recombinant forms, and describe the production and specific detection of a polyclonal anti-Vc-DHDPS antibody. Our results will aid in future studies of Vc-DHDPS, including crystallization, inhibitor screening, and detection of both recombinant and native forms of the enzyme.

2. Materials and methods

2.1. Construction of expression vectors

The *dapA* gene encoding Vc-DHDPS (Vc-*dapA*) was PCR amplified from genomic DNA of the *V. cholerae* O395 strain as the template using the forward (5′-**CATATG**TTTTCAGGAAGTATCGT-3′) and reverse (5′-**GGATCC**TTAATAAATACAGGCTTCAGA-3′) primers. These primers were designed to incorporate *Nde*I and *Bam*HI restriction sites (as highlighted above) at the 5′ and 3′ ends of the Vc-*dapA* gene, respectively. Following PCR amplification using the Platinum PCR SuperMix High Fidelity system (Invitrogen), the resultant product was ligated into the pCR-Blunt-II-TOPO vector (Invitrogen) and transformed into One Shot TOP10 *E. coli* cells (Invitrogen) as per manufacturer's instructions. The resulting plasmid, pCR-Blunt-II-TOPO-Vc-*dapA*, was then digested with the restriction endonucleases *Nde*I and *Bam*HI to release the modified Vc-*dapA* gene. The isolated gene was ligated into corresponding sites of pET-11a and pET-28a vectors using T4 DNA ligase as per manufacturer's instructions (NEB) to produce pET-11a-Vc-*dapA* and pET-28a-Vc-*dapA* (Figs. S1A and S1B) for expressing untagged and His-tagged Vc-DHDPS, respectively. Dideoxynucleotide sequencing [41] was employed to verify the integrity of the Vc-*dapA* insert (Figs. S1C – S1F).

2.2. Overexpression of recombinant Vc-DHDPS

E. coli BL21 (DE3) cells were transformed with pET-11a-Vc-*dapA* and pET-28a-Vc-*dapA*. For untagged Vc-DHDPS, an overnight starter culture at 37 °C (shaking at 160 rpm) was prepared by inoculating 10 mL of Luria Broth (LB) media (containing 75 μg/mL ampicillin) with a single colony of transformed *E. coli* BL21 (DE3) harboring the pET-11a-Vc-*dapA* plasmid. 1 L of LB media (containing 75 μg/mL ampicillin) was inoculated with 10 mL of this starter culture. Cells were grown (shaking at 160 rpm) to an OD_{600} of 0.6 at 37 °C before being treated for 3 h with 1 mM IPTG to induce the expression of recombinant untagged Vc-DHDPS. Cells were harvested by centrifugation (5000 × g, 20 min) at 4 °C and cell pellets stored at −30 °C. The same expression protocol was employed for preparing cell pellets containing recombinant His-tagged Vc-DHDPS, with the only exception being kanamycin (30 μg/mL) was used instead of ampicillin.

2.3. Purification of recombinant Vc-DHDPS

The cell pellet harboring untagged Vc-DHDPS was thawed on ice and resuspended in 10 mL of Buffer 1 (20 mM Tris, 5 mM DTT, pH 8.0). The cell suspension was sonicated on ice using QSonica Q700 sonicator with a 12.7 mm diameter titanium probe for 6–8 cycles (20 s on, 60 s off) at an amplitude of 40 μm. Cell debris was pelleted by centrifugation (48,000 × g, 30 min) at 4 °C and the supernatant was filtered with a 0.45 μM syringe filter. The resulting cell lysate was loaded onto a Q-Sepharose Fast Flow column (100 mL, GE Healthcare) pre-equilibrated in Buffer 1. Bound protein was eluted from the stationary phase using a linear gradient of Buffer 2 (20 mM Tris, 1 M NaCl, 5 mM DTT, pH 8.0) over 10 column volumes. Fractions (7 mL) were collected across the gradient and assessed for DHDPS activity using the *o*-aminobenzaldehyde (*o*-ABA) assay as described previously [42]. Fractions containing DHDPS activity were pooled and crystalline NaCl added incrementally to a final concentration of 2.5 M before loading onto a Phenyl-Sepharose column (60 mL, GE Healthcare) that had been pre-equilibrated with Buffer 3 (20 mM Tris, 2.5 M NaCl, 5 mM DTT, pH 8.0). Bound protein was eluted using Buffer 1 with a decreasing NaCl gradient over 10 column volumes. Fractions (5 mL) were collected across the gradient and examined for DHDPS activity using the *o*-ABA assay. Fractions positive for DHDPS activity were assessed for purity using 12% (w/v) polyacrylamide SDS-PAGE [43]. Fractions containing > 95% pure untagged Vc-DHDPS were pooled, aliquoted, flash frozen in liquid nitrogen, and stored at −80 °C. Prior to experimental analyses, frozen aliquots were thawed on ice, concentrated using a 10,000 Da Amicon filter (Millipore) to approximately 10 mg/mL and passed through a Superose 12 10/300 GL (24 mL, GE Healthcare) column pre-equilibrated in Buffer 4 (20 mM Tris, 150 mM NaCl, 0.5 mM TCEP, pH 8.0). Peak fractions collected during gel filtration (monitored at 280 nm) were used for subsequent kinetic and structural analyses.

The His-tagged Vc-DHDPS cell pellet was thawed on ice and resuspended in 10 mL of Buffer 5 (20 mM Tris, 150 mM NaCl, 20 mM imidazole, 1 mM pyruvate, 0.5 mM TCEP, pH 8.0). Sonication, centrifugation and filtration protocol were followed as described above for untagged Vc-DHDPS. The filtered cell lysate was loaded onto a His-Trap column (5 mL, GE Healthcare) pre-equilibrated with Buffer 5. Once a stable baseline (monitored at 280 nm) was achieved with Buffer 5, bound protein was eluted using a linear gradient with Buffer 6 (20 mM Tris, 150 mM NaCl, 500 mM imidazole, 1 mM pyruvate, 0.5 mM TCEP, pH 8.0) over 10 column volumes. Fractions (7 mL) were collected across the gradient. Peak protein fractions (monitored at 280 nm) were