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Structural characterization of 1-deoxy-D-xylulose 5-phosphate Reductoisomerase from *Vibrio vulnificus*

Nikita K. Ussin[a], Anna M. Bagnell[a], Lesa R. Offermann[a,b], Rawan Abdulsalam[a], Makenzie L. Perdue[a], Patrick Magee[a], Maksymilian Chruszcz[a,*]

[a] Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208, United States
[b] Department of Chemistry, Davidson College, Davidson, NC 28035, United States

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ABSTRACT

Vibrio vulnificus, a gram-negative bacterium, is the leading cause of seafood-borne illnesses and mortality in the United States. Previous studies have identified metabolites 2-*C*-methylerythritol 4-phosphate (MEP) as being essential for *V. vulnificus* growth and function. It was shown that 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr) is a critical enzyme in the viability of *V. vulnificus,* and many other bacteria, as it catalyzes the rearrangement of 1-deoxy-D-xylulose-5-phosphate (Dxp) to 2-*C*-methylerythritol 4-phosphate (MEP) within the MEP pathway, found in plants and bacteria. The MEP pathway produces the isoprenoids, isopentenyl diphosphate and dimethylallyl pyrophosphate. In this study, we produced and structurally characterized *V. vulnificus* Dxr. The enzyme forms a dimeric assembly and contains a metal ion in the active site. Protein produced in *Escherichia coli* co-purifies with Mg^{2+} ions, however the Mg^{2+} cations may be substituted with Mn^{2+}, as both of these metals may be utilized by Dxrs. These findings will provide a basis for the design of Dxr inhibitors that may find application as antimicrobial compounds.

1. Introduction

Vibrio vulnificus is a gram-negative bacterium viable in warm, brackish, coastal water [1]. This particular species of *Vibrio* gains access to the body by ingestion of contaminated raw seafood or through absorption into an open wound that has been in *V. vulnificus* contaminated water [2–4]. Immunocompromised individuals are more susceptible to *V. vulnificus* infections, and the resulting symptoms are more severe in comparison to healthy individuals. *V. vulnificus* is the leading cause of seafood-borne mortality in the United States with fatalities of over 60% due to primary septicemia and over 20% resulting from open wound contamination [1,3,4].

The non-mevalonate or MEP pathway (Fig. 1), that is ubiquitous in most bacteria [5–7], produces the isoprenoids, isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These compounds are essential precursors for vital functions of the bacterial cell, including metabolic functions and membrane stability [8]. This pathway is absent in mammals and therefore is an ideal target for drug development. 1-Deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr) catalyzes the reduction and isomerization of 1-deoxy-D-xylulose 5-phosphate (Dxp) to 2-*C*-methylerythritol 4- phosphate (MEP). In the committed step of the pathway, the enzyme catalyzes the reaction with cofactors NADPH and a divalent cation [7,9–13].

A study seeking to identify essential metabolites in this species of *Vibrio* indicated that knocking down the gene encoding for Dxr rendered the bacteria nonviable [14]. A vast amount of other infections are caused by pathogenic bacteria that also utilize the MEP pathway, including pneumonia, cholera, and gonorrhea [15]. It was shown that knockout of the *dxr* gene in *E. coli* is lethal [16], as well as in *Mycobacterium tuberculosis* [17]. Moreover, Dxr from *Plasmodium falciparum* is a validated target for the development of antimalarial compounds [18,19]. Inhibition of the critical Dxr enzyme can lead to eradication of *V. vulnificus* and a host of other pathogens. Fosmidomycin and FR-900098 (a fosmidomycin analog) are very effective in inhibiting the activity of Dxr [19]. Previous studies have also identified a different class of antibiotics, bisphosphonates, which are also capable of inhibiting the activity of this enzyme [15,20].

Recombinant *V. vulnificus* Dxr (VvDxr) was successfully produced and the protein was structurally characterized. The structure of VvDxr has been determined *via* X-ray crystallography and compared to homologous enzymes originating from *E. coli* (EcDxr), *M. tuberculosis* (MtDxr) and *P. falciparum* (PfDxr).

* Corresponding author at: Department of Chemistry and Biochemistry, University of South Carolina, 631 Sumter Street, Columbia, SC 29208, United States.
E-mail address: chruszcz@mailbox.sc.edu (M. Chruszcz).

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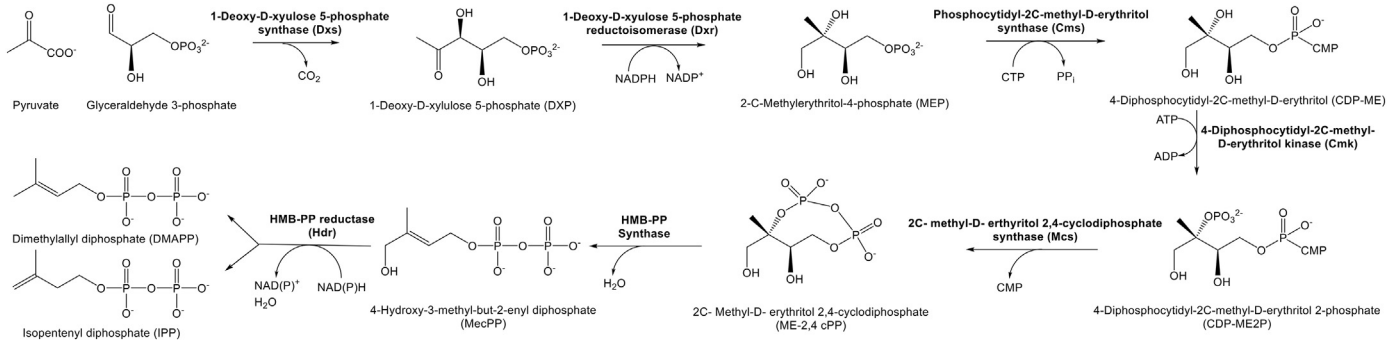

Fig. 1. Non-mevalonate or MEP pathway. This pathway is found in most plants and bacteria and is named for 2-*C*-methyl-D-erythrito 4-phosphate (MEP) [7,11,12].

2. Materials and methods

2.1. Protein production and purification

Synthetic VvDxr gene was ordered from DNA 2.0 (Menlo Park, CA) in expression vector pJexpress411. The construct included a sequence coding for a TEV cleavable, N-terminal polyhistidine tag (MHHHHHH-HSSGVDLGTENLYFQ↓SGSG). This plasmid was transformed into *E. coli* strain BL21 (DE3) and then grown overnight on Luria-Bertani (LB) agar plates supplemented with kanamycin. Protein was produced using a protocol published previously [21]. Briefly, cultures were grown to an OD_{600} of 0.8 then induced with 400 μM isopropyl β-D-1-thiogalacto-pyranoside (IPTG) and grown overnight at 289 K. Culture media were centrifuged to pellet cells. Cells were resuspended in 30 mL of lysis buffer (50 mM Tris, 500 mM NaCl, 10 mM imidazole, 2% glycerol, and 20 mM 2-mercaptoethanol, pH 7.5) and lysed by sonication. The homogenate was centrifuged, and supernatant was loaded onto Ni-NTA agarose (Thermo Scientific, Wilmington, DE) media equilibrated with the lysis buffer. The column was washed again with 10 column volumes of solution containing: 50 mM Tris, 500 mM NaCl, 30 mM imidazole, 2% glycerol, and 20 mM 2-mercaptoethanol, pH 7.5. The protein was then eluted from the column using elution buffer (50 mM Tris, 500 mM NaCl, 250 mM imidazole, 2% glycerol, and 20 mM 2-mercaptoethanol). Fractions containing protein were pooled and dialyzed in solution composed of 10 mM Tris, 150 mM NaCl, 5 mM 2-mercaptoethanol (pH 7.4) overnight. Protein was concentrated using an Amicon Ultra concentrator (Millipore) with a 10 kDa molecular weight cut-off and purified on a Superdex 200 column attached to an ÄKTA Pure FPLC system (GE Healthcare). A solution composed of 10 mM Tris-HCl and 150 mM NaCl at pH 7.4 was used for gel filtration of VvDxr. Following gel filtration, fractions containing protein were pooled and concentrated to approximately 9 mg/mL. Protein concentration was determined using the Bradford method [22]. His-tag cleavage was accomplished first by diluting uncut VvDxr to 1.5 mg/mL with gel filtration buffer then, then adding His-tagged TEV protease (protease-to-protein ratio of 1:100 (w/w)). Solution containing VvDxr and TEV was kept overnight at 277 K. TEV protease was removed using a Ni-NTA agarose column. VvDxr with cleaved His-tag was purified again on the Superdex 200 column. After gel filtration, fractions containing protein were pooled and concentrated to 12 mg/mL. His-tag cleavage was confirmed using mass spectrometry.

2.2. Crystallization

Crystallization of VvDxr was performed at 298 K. All initial crystallization experiments were performed using the hanging drop vapor diffusion method utilizing the Qiagen EasyXtal 15 well plates (Hilde, DE) and uncleaved protein. Initial screening was performed using Wizard Classic I and II screens (Rigaku, Bainbridge Island, WA). In the second round of crystallization, protein with the purification tag removed was used. In this case both hanging and sitting drop vapor diffusion methods were utilized. The sitting drop vapor diffusion experiments were performed using NeXtal plates (Qiagen, Chatsworth, CA). Index screen (Hampton Research, Aliso Viejo, CA) was used in addition to Wizard I and II screens.

2.3. Data collection, processing and structure determination

VvDxr crystals were cryo-protected using well solution with addition of ~20% v/v ethylene glycol and cryo-cooled in liquid nitrogen prior to diffraction experiments. Diffraction experiments were performed at 22BM and 22ID of the Southeast Regional Collaborative Access Team (SER-CAT) and 19ID of the Structural Biology Center [23] at the Advanced Photon Source (APS) of Argonne National Laboratory (ANL). Data processing was performed using the HKL-2000 package [24]. Details for the data-collection statistics are shown in Table 1.

VvDxr structures were determined using HKL-3000 [25], MOLREP [26] and selected programs from the CCP4 suite [27]. Molecular replacement was used for phasing. The first, *apo*-form of VvDxr (PDB ID: 5KQO) was determined using the *E. coli* Dxr homolog (PDB ID: 1Q0L) as a starting model. Other structures of VvDxr were determined using 5KQO as their starting model. HKL-3000 and REFMAC [28] were used to refine all structures, followed by rebuilding and validation using COOT [29] and Molprobity [30]. All coordinates, together with structure factors, were deposited into the Protein Data Bank (PDB) and were given the following accession codes: 5KQO (uncleaved His-tag); 5KS1 (His-tag cleaved; soaked with fosmidomycin and Mn^{2+}); 5KRR (His-tag cleaved; soaked with Mn^{2+} and FR900098); 5KRY (His-tag cleaved with phosphate-free condition); and 5KRV (uncleaved His-tag; co-crystallized with Arginine). Table 1 provides a summary of the model refinement and validation.

2.4. Various other methods

PDBePISA was used to calculate interface areas in dimeric Dxr assemblies [31]. PDBeFOLD [32] and DALI [33] were used to find structural homologs of VvDxr. Clustal Omega [34] and ESPript [35] were used to prepare the figure with sequence alignment. Pymol [36] was used for figures showing molecular models.

3. Results

3.1. Overexpression and purification of VvDxr

VvDxr was produced and purified in high quantities, and on average we were able to produce ~150 mg of the protein per 1 L of culture. Cleavage of the His-tag was also successful, and the protein in both His-tagged and His-tag cleaved forms were most stable in the pH range 5.5–6.5 (1 M NaCl) as determined by Differential Scanning Fluorimetry with maximum melting temperatures of ~60 °C [21]. Generally, VvDxr is more stable in solution with a relatively high salt concentration. This is not surprising taking into account that *V. vulnificus* is a halophilic