Journal of Structural Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Structural Biology

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

Comparative structural and enzymatic studies on *Salmonella typhimurium* diaminopropionate ammonia lyase reveal its unique features

G. Deka[a], S. Bisht[a], H.S. Savithri[b], M.R.N. Murthy[a,*]

[a] Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India
[b] Biochemistry Department, Indian Institute of Science, Bangalore 560012, India

ARTICLE INFO

Keywords:
Diaminopropionate ammonia lyase
PLP
Phosphate
Disulfide bond
Metal ion dependent activity
X-ray structure

ABSTRACT

Cellular metabolism of amino acids is controlled by a large number of pyridoxal 5′-phosphate (PLP) dependent enzymes. Diaminopropionate ammonia lyase (DAPAL), a fold type II PLP-dependent enzyme, degrades both the D and L forms of diaminopropionic acid (DAP) to pyruvate and ammonia. Earlier studies on the *Escherichia coli* DAPAL (*Ec*DAPAL) had suggested that a disulfide bond located close to the active site may be crucial for maintaining the geometry of the substrate entry channel and the active site. In order to obtain further insights into the catalytic properties of DAPAL, structural and functional studies on *Salmonella typhimurium* DAPAL (*St*DAPAL) were initiated. The three-dimensional X-ray crystal structure of *St*DAPAL was determined at 2.5 Å resolution. As expected, the polypeptide fold and dimeric organization of *St*DAPAL is similar to those of *Ec*DAPAL. A phosphate group was located in the active site of *St*DAPAL and expulsion of this phosphate is probably essential to bring Asp125 to a conformation suitable for proton abstraction from the substrate (D-DAP). The unique disulfide bond of *Ec*DAPAL was absent in *St*DAPAL, although the enzyme displayed comparable catalytic activity. Site directed mutagenesis of the cysteine residues involved in disulfide bond formation in *Ec*DAPAL followed by functional and biophysical studies further confirmed that the disulfide bond is not necessary either for substrate binding or for catalysis. The activity of *St*DAPAL but not *Ec*DAPAL was enhanced by monovalent cations suggesting subtle differences in the active site geometries of these two closely related enzymes.

1. Introduction

Diaminopropionate ammonia lyase (DAPAL, encoded by *ygeX* gene) is a fold type II pyridoxal 5′-phosphate (PLP) dependent enzyme (Rao et al., 1970, Nagasawa et al., 1988, Vijayalakshmi et al., 1975). DAPAL degrades with comparable efficiency both D and L isomers of the nonstandard amino acid diaminopropionic acid (DAP) to pyruvate and ammonia (Nagasawa et al., 1988, Khan et al., 2003). DAP is a precursor in the biosynthesis of the neurotoxins 3-oxalyl and 2,3-dioxalyl DAP (O-DAP) (Cheema et al., 1969, Spencer et al., 1986). O-DAP is abundant in the seeds of a drought resistant legume, *Lathyrus sativus*. Consumption of this legume seeds in large quantities causes neurolathyrism and osteolathyrism in humans (Cheema et al., 1969, Spencer et al., 1986). It might be possible to reduce the neurotoxin content of the *Lathyrus sativus* seeds by engineering transgenic plants that over express DAPAL. DAP is also a constituent of non-ribosomal peptide antibiotics such as capreomycin, zwittermicin and viomycin (Haskell et al., 1952, Wang and Gould, 1993, Zhao et al., 2008).

Escherichia coli DAPAL (*Ec*DAPAL) and *Salmonella typhimurium* DAPAL (*St*DAPAL) share only 50% sequence identity whereas most other homologous enzymes belonging to these two bacterial species share much higher sequence identity (> 85%) (Kalyani et al., 2012). Therefore, it is of interest to investigate structural and functional differences between these two enzymes. The crystal structure of *Ec*DAPAL has been determined in its PLP bound (holo), unbound (apo) forms as well as in a form bound with the catalytic intermediate aminoacrylate (Bisht et al., 2012). Biochemical studies on the active site mutants of *St*DAPAL coupled with structural information obtained from *Ec*DAPAL had suggested that DAPAL might follow a two base mechanism for the degradation of the two stereo-isomers of DAP (D or L-DAP) (Bisht et al., 2012, Kalyani et al., 2013).

The crystal structure of *Ec*DAPAL revealed the presence of a disulfide bond not far from the active site (Bisht et al., 2012). Biochemical studies in solution using Ellman's reagent also established the presence of the disulfide bond (Kalyani et al., 2013). Other fold type II PLP-dependent enzymes do not possess an equivalent disulfide bond. Instead, a bound metal ion has been observed in these enzymes. Based on these observations, it was suggested that the disulfide bond in *Ec*DAPAL may

* Corresponding author.
E-mail address: mrn@iisc.ac.in (M.R.N. Murthy).

https://doi.org/10.1016/j.jsb.2017.12.012
Received 12 October 2017; Received in revised form 22 December 2017; Accepted 26 December 2017

replace the role of metal ion found in other fold type II PLP dependent enzymes and may be essential for the structural integrity of a segment corresponding to residues 261–295 close to the active site that could control the diffusion of substrates and products (Bisht et al., 2012). In contrast to these observations, solution studies suggested that that there is no disulfide bond in *St*DAPAL (Kalyani et al., 2013) although the two cysteines (Cys265 and Cys291) involved in the formation of disulfide bond in *Ec*DAPAL are conserved in *St*DAPAL (Cys271 and Cys299). However, in the absence of the crystal structure, the conformational state of the loop in *St*DAPAL corresponding to residue 261–295 of *Ec*-DAPAL was unknown and hence its relation to catalysis could not be discerned.

The activity of several fold type II PLP dependent enzymes such as D-serine deaminase (DSD) and serine recamase (SR) is enhanced in the presence of metal ions such as Na^+/K^+ or Mg^{2+} (Bharath et al., 2011, Smith et al., 2010). Examination of the enzymatic properties of *St*DAPAL showed that the activity is enhanced by monovalent metal ions (K^+ and Na^+) but not by divalent metal ions (Kalyani et al., 2013). Surprisingly, the enzymatic activity of *Ec*DAPAL was not metal ion dependent. With the view of examining the role of disulfide bond in the catalytic activity of DAPAL and to understand the importance of metal ions in the activity of *St*DAPAL and other fold type II PLP-dependent enzymes, structural studies on *St*DAPAL and biochemical studies on the disulfide bond mutants of *Ec*DAPAL were initiated.

In this communication, we present the crystal structure of *St*DAPAL and functional and biophysical characterization of two disulfide bond mutants of *Ec*DAPAL. Detailed analysis of the activities and structures of these proteins and comparison with other fold type II PLP enzymes provide new insights on the structural and functional features of DAPAL.

2. Experimental procedures

2.1. Cloning, over-expression and purification

The gene encoding *St*DAPAL was cloned, over-expressed and purified as described previously (Kalyani et al., 2013). The purity of the protein was ascertained by SDS-PAGE. MALDI-TOF mass spectrometric data indicated that the protein was purified as the full length polypeptide with a molecular mass of 45.22 kDa. Unlike *St*DAPAL, *Ec*DAPAL and its C291S and C265S mutants did not have a hexahistidine tag. Therefore, these proteins were purified in the absence of metal ions as described earlier (Bisht et al., 2012; Kalyani et al., 2013). Briefly, *Ec*-DAPAL and its disulfide mutants (C265S, C291S) were eluted from a DEAE-cellulose column using 20 mM Tris-HCl, pH 6.0 and further purified using a Superdex-200 gel filtration column previously equilibrated with the same buffer at pH 7.5. The fractions containing the proteins were pooled, concentrated and used for further studies. *St*DAPAL, *Ec*DAPAL and all mutants of *Ec*DAPAL were found to exist in their dimeric form by gel filtration chromatography.

2.2. Site directed mutagenesis

Single site mutants of *Ec*DAPAL targeting residues Cys265 and Cys291 were constructed by site directed mutagenesis. *Ec*DAPAL cloned in pRSET C vector was used as the template for the generation of mutants. The primers used for introducing site specific mutations and the restriction sites inserted in the sense and antisense primers for the easy screening of the mutants are shown in Table 1. All the mutants were confirmed by sequencing.

2.3. Crystallization

Crystals of *St*DAPAL appeared 15–20 days after setting up micro-batch method of crystallization in drops consisting of 2 µl of protein (20 mg/ml) and 2 µl of crystallization condition containing 20% (w/v) PEG 4000, 20% (v/v) 2-propanol, 100 mM sodium citrate pH 5.6 (condition corresponding to JBScreen Basic 2/C2). However, these crystals had thin plate like morphology, very delicate and did not diffract X-rays to resolutions adequate for structural studies (Fig. S1A). By optimizing the crystallization condition, better crystals (Fig. S1B) of size 0.3–0.4 mm were obtained after 10–15 days from solutions containing 30% (w/v) PEG 4000, 20% (v/v) 2-propanol, 100 mM sodium citrate pH 5.6. The crystals used for diffraction experiments were cryocooled in liquid nitrogen after soaking in crystallization buffer containing 20% (v/v) ethylene glycol as the cryoprotectant for a few seconds. The crystal used for data collection diffracted X-rays to 2.5 Å resolution.

Table 1
Primers used for generating site specific *Ec*DAPAL mutants.

Mutant	Primer	Description
C265S	GACAAAGCTGACAGTATTTATCGCTCCGGA GTCAAAGGCG	BspEI site TCCGGA
	GACAAAGCTGACTGTATTTATCGCTCCGGC	Introduced
C291S	GTCAAAGGCG GGCAGGCCTGGCCAGTGGCGAACC	MscI site TGGCCA
	GGCAGGCCTGGCCTGTGGCGAACC	Introduced

2.4. Data collection, structure determination and analysis

X-ray diffraction data were collected using synchrotron radiation at BM14 beam line of ESRF, Grenoble on a MarCCD detector. The crystal was maintained at 100 K using an Oxford Cryosystems apparatus. Radiation of wavelength 0.95 Å was used for collecting the data. A total of 180 frames, each with 1° oscillation angle and 2 s exposure time, were recorded. Diffraction data thus obtained were processed using *iMosflm* (Battye et al., 2011) and scaled using *AIMLESS* (Evans and Murshudov, 2013) from the *CCP4* program suite (Winn et al., 2011). The program *Phaser* (McCoy et al., 2007) of the *CCP4* suite was used to obtain a molecular replacement structure solution for *St*DAPAL using *Ec*DAPAL (PDB code: 4D9G, sequence identity 50%) as the phasing model. Rigid body refinement followed by several rounds of manual model adjustment using COOT (Emsley et al., 2002) and restrained refinement of positional parameters with Refmac5 (Murshudov et al., 2011) of CCP4 suite were carried out to improve the model.

The geometries of the final models were validated using PROCHECK (Laskowski et al., 1993). Average B-factors for protein atoms, water molecules and ligand atoms were calculated using the BAVERAGE program of the CCP4 suite. PISA (Krissinel and Henrick, 2007) was used for the analysis of interfaces and identification of interfacial residues. DALI server (Holm and Rosenstrom, 2010) was used for the identification of structural homologs in the PDB. Structural superposition with homologous protein folds was achieved and root mean square deviation (RMSD) between structurally equivalent Cα atoms were calculated using the program ALIGN (Cohen, 1997) and SSM superpose (Krissinel and Henrick, 2004) of COOT (Emsley et al., 2002). Figures were prepared using the program PyMOL (DeLano, 2002).

2.5. Activity assay and spectral studies

A coupled enzyme assay using lactate dehydrogenase (LDH) as the second enzyme was used to estimate the amount of the pyruvate produced in the reaction catalyzed by DAPAL. LDH converts pyruvate to lactate with concomitant conversion of NADH to NAD^+. As the reaction proceeds, absorbance at 340 nm decreases due to depletion of NADH. All assays were carried out in 50 mM potassium phosphate buffer, pH 7.5 containing 10 µM PLP. The assays were performed in 1 mL reaction mixture at 37 °C for a time period of 300 s. One unit of DAPAL activity was defined as the amount of enzyme required to convert 1 µmol of