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Structural and biochemical studies on the role of active site Thr166 and Asp236 in the catalytic function of D-Serine deaminase from *Salmonella typhimurium*

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ABSTRACT

D-Serine deaminase (DSD) degrades D-Ser to pyruvate and ammonia. Uropathogenic bacteria survive in the toxic D-Ser containing mammalian urine because of DSD activity. The crystal structure of the apo form of *Salmonella typhimurium* DSD (*St*DSD) has been reported earlier. In the present work, we have investigated the role of two active site residues, Thr166 and Asp236 by site directed mutagenesis (T166A and D236L). The enzyme activity is lost upon mutation of these residues. The 2.7 Å resolution crystal structure of T166A DSD with bound PLP reported here represents the first structure of the holo form of *St*DSD. PLP binding induces small changes in the relative dispositions of the minor and major domains of the protein and this inter-domain movement becomes substantial upon interaction with the substrate. The conformational changes bring Thr166 to a position at the active site favorable for the degradation of D-Ser. Examination of the different forms of the enzyme and comparison with structures of homologous enzymes suggests that Thr166 is the most probable base abstracting proton from the Cα atom of the substrate and Asp236 is crucial for binding of the cofactor.

1. Introduction

D-amino acids such as D-Ala and D-Glu are components of bacterial cell walls [1]. Although D-Ser is toxic, most bacteria survive and propagate in D-Ser containing media by degrading D-Ser to pyruvate and ammonia (Scheme 1), a reaction catalyzed by D-Serine deaminase (DSD, encoded by dsdA gene). Uropathogenic bacteria such as *S. saprophyticus* and *E. coli* (UPEC) survive in D-Ser containing urine due to the action of DSD [2] [3]. D-Ser also plays an important role in regulating the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor [4].

DSD (EC 4.3.1.18) belongs to fold type II or tryptophan synthase β (TRPSβ) family of pyridoxal 5′ phosphate dependent (PLP) enzymes. PLP is a versatile cofactor for a wide variety of enzymes that carry out elimination, replacement, transamination, decarboxylation and racemization reactions with amino acid substrates [5]. DSD has been biochemically characterized from *E. coli* [6,7], *Klebsiella* [8], *S. cerevisiae* [9] and chicken [10]. Crystal structures of DSD from *E. coli* (PDB code: 3SS7 [11]), *P. xenovorans* (PDB code: 3GWQ, Joint Center for Structural Genomics) and *S. typhimurium* (PDB code: 3R0Z [12]) have been determined. The *E. coli* DSD (*Ec*DSD) and *S. typhimurium* DSD (*St*DSD) are monomeric proteins unlike most of the fold type II PLP dependent enzymes such as 2, 3-Diaminopropionate ammonia lyase (DAPAL [13]), O-acetylserine sulfhydrylase (OASS [14]), which are dimers. Crystals of wild type *St*DSD (Wt-*St*DSD, PDB code: 3R0Z) and SeMet incorporated DSD co-crystallized in presence of isoserine (SeMet-*St*DSD, PDB code: 3R0X) belong to the space groups C2 and $P2_12_12$, respectively [12]. The structures of both these crystal forms represent the apo form of the enzyme. These structures suggested that the enzyme undergoes a large conformational change from an open to a closed conformation upon interaction with isoserine [12]. Based on these structures, it was suggested that Thr166 might be responsible for abstraction of proton from Cα atom of D-Ser in the catalytic reaction. It was also proposed that Asp236 might play a role in external aldimine formation [12]. However, because of lack of crystal structure of *St*DSD bound to the cofactor PLP, these proposals could

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Scheme 1. The degradation of D-Ser to pyruvate and ammonia by D-Serine deaminase, a PLP dependent enzyme.

not be validated further.

In this manuscript, we present the X-ray crystal structure of an active site mutant (T166A) of *St*DSD; representing the first PLP-bound (holo) form of the enzyme. We have carried out biochemical studies on the T166A and D236L mutants of DSD to understand the role of Thr166 and Asp236 in the function of DSD. The structural features that are important for catalysis are discussed in the light of these structural and biochemical investigations.

2. Materials and methods

2.1. Cloning, over-expression and purification of active site mutants

The gene for *St*DSD cloned in pET21b vector was used as the template for the generation of single site mutants [12]. Two active site residues Thr166 and Asp236 were targeted for mutagenesis (shown in red italics) using single primer extension method [15]. The primers were designed so as to contain a restriction site (shown in green italics) by choosing appropriate degenerate codons. The mutants were further confirmed by sequencing.

D236L: ctgtttctttatcgac*ctc**gag*aactctcgcacgc

T166A: gccgtcggttca*gcc**ggc*aatctgg

The two active site mutants of *St*DSD were over-expressed and purified using the protocol found suitable for the wild type enzyme [12]. The purified proteins corresponded to a size of 49 kDa when examined on a 12% SDS-PAGE.

2.2. Crystallization of StDSD

The purified proteins were incubated with 0.1% of n-octyl-β-glucopyranoside prior to setting up crystallization. Initial trials of crystallization were carried out at 298 K using the hanging drop method under the conditions found suitable for the wild type enzyme [12]. Crystals of mutant proteins (T166A and D236L) could not be obtained under this condition or with small variations of the condition. Therefore, screening for suitable conditions was carried out using a number of commercially available screens including Hampton Crystal screens 1 and 2, Index screens 1 and 2, Jena Basic screens 1–4 and Jena classic screens 1–10. No hits were obtained for D236L. However, crystals of T166A were obtained from 20% w/v polyethylene glycol 4,000, 100 mM MES pH 6.5, 600 mM sodium chloride (JBscreen classic 3/C2). These crystals were fragile and difficult to mount. The diffraction data obtained from the best crystal was used for structural studies.

2.3. X-ray diffraction data collection

A single crystal of *St*DSD-T166A was mounted on a cryo-loop and frozen in liquid nitrogen. X-ray diffraction data extending to 2.7 Å were recorded using a CCD image plate detector while maintaining a temperature of 100 K at the BM14 beamline at ESRF, Grenoble. The data were processed using the program iMOSFLM and scaled using SCALA of the CCP4 suite [16]. The crystal belonged to the space group C2 with unit cell parameters of a = 108.31 Å, b = 46.14 Å, c = 99.28 Å and β = 100.8°. Although the space group is the same, the unit cell parameters for the T166A mutant were different from those of the wild type enzyme (PDB code: 3R0Z) for which a = 100.02 Å, b = 46.79 Å, c = 100.04 Å and β = 93.75°.

2.4. Structure determination and refinement

The structure of *St*DSD-T166A mutant was determined by molecular replacement using Wt-*St*DSD (PDB code: 3R0Z) as the phasing model. All hetero atoms and water molecules of the phasing model were removed to avoid model bias. The solution obtained by PHASER [17] was first subjected to rigid body refinement and then subjected to positional refinement using Refmac5 of CCP4 suite [18]. Each cycle of refinement was followed by manual model building using COOT [19].

2.5. Spectral studies and activity assays

Spectral studies were carried out to monitor the formation of internal aldimine as well as the product pyruvate using a Jasco UV–Visible V-630 spectrophotometer. 1 mg/ml of protein in 50 mM HEPES pH 7.5, 100 mM NaCl buffer were used for the study. The reactions were initiated by addition of 1 mM of D-Ser and spectra were recorded in the range of 300–550 nm as a function of time.

The specific activities of the Wt-*St*DSD as well as the two active site mutants (T166A and D236L) were estimated using 2,4-dinitrophenyl hydrazine (DNPH) method by measuring the α-keto acid released during the reaction as described previously [12]. The reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.5), 50 μM PLP, 100 μM of the substrate D-Ser and 50 ng of enzyme.

3. Results

3.1. X-ray crystal structure determination of StDSD-T166A

All the diffraction datasets collected for T166A-DSD mutant crystals contained spots from polycrystalline contaminations making meaningful data processing very difficult. Only a single data set collected on a crystal of T166A which contained multiple ice rings that could be processed (Supplementary Fig. S1) by excluding the contaminating rings [20]. Because of the ice rings, the quality of the processed data was low. This is reflected in the data statistics (Table 1).

The structure of *St*DSD-T166A was determined by molecular replacement using the wild type structure as the phasing model from which all non-protein atoms were removed. The solution obtained was refined to a final R_{work} and R_{free} of 28.91% and 34.85%, respectively, using REFMAC5 of CCP4 suite [18]. The refinement statistics is listed in Table 2.

3.2. Quality of the model

Despite the limited resolution (2.7 Å) and data quality, a model (residues 2–439) of the mutant protein *St*DSD-T166A could be built into the final electron density map with 99.4% of the residues in the allowed regions of the Ramachandran map and only two residues (0.60%) in the disallowed region. Continuous electron density could be traced for most of the main chain as well as the side chain atoms with the exception of a few residues (69–73, 212–227 and the C-terminal hexa-histidine tag) where electron densities were either missing or fragmented. These residues have not been included in the final model.