FEBS Letters 589 (2015) 3842–3847

FEBS Letters

journal homepage: www.FEBSLetters.org

Structural basis for an atypical active site of an L-aspartate/glutamate-specific racemase from *Escherichia coli*

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ARTICLE INFO

Article history:
Received 7 August 2015
Revised 3 November 2015
Accepted 3 November 2015
Available online 7 November 2015

Edited by Stuart Ferguson

Keywords:
Asp/glu racemase
L-Form specific racemase
Amino acid enantiomer
Structure

ABSTRACT

We determined the crystal structure of *Ec*L-DER to elucidate protein function and substrate specificity. Unlike other asp/glu racemases, *Ec*L-DER has an unbalanced pair of catalytic residues, Thr83/Cys197, at the active site that is crucial for L- to D-unidirectional racemase activity. *Ec*L-DER exhibited racemase activity for both L-glutamate and L-aspartate, but had threefold higher activity for L-glutamate. Based on the structure of the *Ec*L-DER^{C197S} mutant in complex with L-glutamate, we determined the binding mode of the L-glutamate substrate in *Ec*L-DER and provide a structural basis for how the protein utilizes L-glutamate as a main substrate. The unidirectionality, despite an equilibrium constant of unity, can be understood in terms of the Haldane relationship.

1. Introduction

D-Amino acids play significant physiological roles despite their rare occurrence in nature. In human plasma, free D-serine, D-alanine, and D-proline have been reported [1]. Interestingly, D-serine in mouse and rat brains was identified as a mediator or transmitter in the nervous system [2–4]. In bacteria, D-amino acids such as D-glutamic acid and D-alanine are building blocks of the peptidoglycan layer, an essential component of the bacterial cell wall that determines cell morphology and provides resistance to osmotic rupture [5]. They are also constituents of microbial secondary metabolites such as the immunosuppressive agent cyclosporin A and the antibiotic gramicidin S [6–8]. It is important to uncover the reaction mechanism and characteristics of the amino acids, since D-amino acids are produced from L-amino acids by a racemase that catalyzes the stereo chemical inversion and some racemases can be used for the industrial production [9] of D-amino acids that are used as precursors for pharmaceuticals [10]. Particularly, an understanding of the molecular mechanism of glutamate racemase could be useful in the design of new antibiotics as the disruption of peptidoglycan biosynthesis is fatal to bacteria [11].

Amino acid racemases can be classified into two groups depending on the presence or absence of a cofactor. Alanine and arginine racemases require pyridoxal 5′-phosphate (PLP) [12,13], which forms a Schiff base with the amino acid substrate. On the contrary, aspartate racemase [14–16] or the glutamate racemase from *Escherichia coli* [17] or *Lactobacillus* [18,19] are PLP-independent enzymes. In the sequence of the PLP-independent amino acid racemases from various organisms, a pair of cysteine residues is highly conserved. Studies using chemical modification and site directed mutagenesis revealed that these conserved cysteines are utilized as the catalytic acid/base and suggest that the "two-base" mechanism catalyzes racemization [14,20–23]. One enzyme base deprotonates an α-carbon of the amino acid and the second base, in its acidic form, transfers a proton to the opposite site of the α-carbon [20].

This mechanism is supported by the determination of the crystal structure of GluR [24], AspR [25], and DapE [26]. These possess two superimposable α/β domains, the N- and C-terminal domains, and they form a pseudo mirror-symmetry in the active site. The symmetrical arrangement between the catalytic cysteines of the N- and C-terminal domains was able to function as a catalytic acid–base pair [25]. It was also reported that the catalytic cysteine of the N-terminal domain is responsible for the deprotonation of

Author contributions: J.W.A. performed experiments, J.H.C. analyzed data and wrote manuscript, and K.J.K. designed experiments and wrote manuscript.

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http://dx.doi.org/10.1016/j.febslet.2015.11.003
0014-5793/

D-glutamate, while that of the C-terminal domain deprotonates L-glutamate.

Based on genomic analysis of *E. coli*, the gene product of *B21_02649* is classified as the asp/glu racemase superfamily and is annotated primarily as a putative racemase. In this study, based on structural and biochemical experiments, we annotate the B21_02649 protein as *Ec*L-DER, an L-aspartate/glutamate-specific racemase. Moreover, we provide a structural basis for how *Ec*L-DER exhibits racemase activity solely for L-glu/asp as a substrate.

2. Materials and methods

2.1. Protein preparation

The B21_02649 coding gene was amplified from the chromosomal DNA of an *E. coli* strain BL21(DE3) using polymerase chain reaction (PCR) with the following primers: sense, 5′-GCGCGCATATGAAAACAATTGGTTTGC-3′ and antisense, 5′-TATATCTCGAGCGACAGCATAAAAGCGAC-3′. The amplified PCR fragment was then subcloned into a pET30a vector (Novagen) with *NdeI* and *XhoI* restriction enzymes. The B21_02649 protein, containing a 6X His tag at the C-terminus, was over-expressed in the *E. coli* strain BL21(DE3). Cells were cultured in an LB medium containing 50 ng/ml kanamycin at 37 °C, until reaching an absorbance of 0.6 at 600 nm. After induction with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 18 h at 18 °C, cells were collected by centrifugation at 5000×*g* for 20 min at 4 °C. The cell pellet was resuspended in ice-cold buffer A (40 mM Tris–HCl, pH 8.0, 10 mM β-mercaptoethanol) and disrupted by ultrasonication. Cell debris was removed by centrifugation at 12,000×*g* for 50 min, and lysate was bound to Ni–NTA agarose (QIAGEN). After washing with buffer A containing 10 mM imidazole, the bound proteins were eluted with 300 mM imidazole in buffer A. Further purification was performed using Sephacryl S-300 HR (GE Healthcare). The purified protein was >95% pure, as determined by Coomassie blue stained SDS–PAGE, and concentrated to 50 mg/ml in a buffer consisting of 20 mM Tris–HCl pH 8.0 and 5 mM β-mercaptoethanol. The B21_02649 mutants were prepared using site-directed mutagenesis and purified following a procedure similar to that described for the wild-type protein.

2.2. Crystallization

Crystallization of the purified protein was initially performed with crystal screening kits (Hampton Research Co. and Emerald Biostructures Co.) with 25 mg/ml and by using the hanging-drop vapor-diffusion method at 20 °C. Suitable crystals were grown by the hanging-drop method using 0.3 M ammonium nitrate and 14–16% PEG 3350 at 20 °C, and crystals reached their maximal sizes within 7–15 days with dimensions of approximately 0.1 × 0.1 × 0.2 mm. For data collection, 30% (w/v) glycerol was added to the crystallizing precipitant as a cryoprotectant, and the crystals were immediately placed in a 100 K nitrogen-gas stream. X-ray diffraction data were collected at a resolution of 1.8 Å at the 7A beamline of the Pohang Accelerator Laboratory (PAL, Korea). For the structure in complex with L-glutamate, the *Ec*L-DER^{C197S} mutant crystals were prepared with similar crystallization conditions, and 20 mM L-glutamate was soaked into the mutant crystal for 5 min.

2.3. Structure determination

The data were indexed, integrated, and scaled using the HKL2000 suite [27]. The apo-form of crystals belonged to $C222_1$ space group, with the unit cell parameters $a = 82.17$, $b = 147.86$, and $c = 82.18$ Å. With two B21_02649 molecules in the asymmetric unit, the crystal volume per unit of protein weight was 2.40 Å^3 Da^{-1}, corresponding to a solvent content of 48.81% [28]. L-Glu complex crystal belonged to $P2_1$ space group, with the unit cell parameters $a = 67.53$, $b = 81.25$, and $c = 83.96$ Å with $\beta = 111.39°$.

The apo-form of the structure was determined by molecular replacement with the program Molrep [29] from CCP4 suite [30] using the crystal structure of a putative aspartate racemase from *Pyrococcus horikoshii* OT3 (PDB code: 1JFL) [25] as a search model. Model building was performed using the program Coot [31], and refinement was performed with CCP4 Refmac5 [32]. The structure in complex with L-Glu was determined by molecular replacement using the apo-form of B21_02649 as a search model. Model building and structure refinement were performed using the same procedure as the apo-form structure. X-ray diffraction and structure refinement statistics are summarized in Table 1. The atomic coordinates and structure factors of the apo-form and L-glutamate-complex form were deposited in the Protein Data Bank with pdb codes of 5ELL and 5ELM, respectively.

2.4. Racemase activity assay

A racemase activity assay was performed based on the circular dichroism spectrum to distinguish D- and L-enantiomers of amino acids [18,33]. A solution (3 ml) containing D/L-glutamic acid or L-aspartic acid in 10 mM potassium phosphate buffer (pH 8.2) was incubated at 4 °C for 1 h. To start the reaction, the enzyme (100 ng/ml) was added to the mixture, followed by incubation at 37 °C for 30 min. The enzyme reaction was stopped by boiling the reaction mixture for 5 min. Ellipticity was monitored in a 1 cm-path length cylindrical cell at 25 °C using a Jasco J-1500 circular dichroism spectrometer equipped with a thermostated cuvette holder. The scan rate and bandwidth were set to 200 nm/min and 2.0 nm, respectively. Spectra were recorded between 200 and 220 nm by 0.5 nm carving. The machine sensitivity was set to 20–50 mdegrees, and the resulting spectra were treated without smoothing.

Table 1
Statistics for data collection and structure refinement.

	Apo	Complex with L-Glu
Data collection		
Space group	$C222_1$	$P2_1$
Cell dimensions		
a, *b*, *c* (Å)	82.17, 147.86, 82.18	67.53, 81.25, 83.96
α, β, γ (°)	90.00	90.00, 111.39, 90.00
Resolution (Å)	50.00–1.80 (1.83–1.80)*	50.00–2.00 (2.11–2.00)*
R_{merge} (%)	8.9 (32.4)	9.4 (21.3)
I/σ	23.53 (3.44)	6.0 (2.9)
Completeness (%)	96.3 (90.8)	98.3 (94.9)
Redundancy	4.8 (2.9)	3.3 (3.1)
Refinement		
Resolution (Å)	25.70–1.80	29.32–2.00
No. reflections	42,538	53,262
R_{work} (%)/R_{free} (%)	14.7/18.9	17.3/23.6
No. atoms	4247	7990
Protein	3617	7355
L-Glutamic acid		30
Water	616	589
β-Mercaptoethanol	4	
Glycerol	6	12
Nitric acid	4	4
Averaged *B*-factors	21.8	21.2
R.m.s. deviations from ideal value		
Bond lengths (Å)	0.019	0.017
Bond angles (°)	1.818	1.738

* Values in parentheses are for highest-resolution shell.