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Lactobacillus brevis CGMCC 1306 glutamate decarboxylase: Crystal structure and functional analysis

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ABSTRACT

Glutamate decarboxylase (GAD), which is a unique pyridoxal 5-phosphate (PLP)-dependent enzyme, can catalyze α-decarboxylation of L-glutamate (L-Glu) to γ-aminobutyrate (GABA). The crystal structure of GAD in complex with PLP from *Lactobacillus brevis* CGMCC 1306 was successfully solved by molecular-replacement, and refined at 2.2 Å resolution to an R_{work} factor of 18.76% (R_{free} = 23.08%). The coenzyme pyridoxal 5-phosphate (PLP) forms a Schiff base with the active-site residue Lys279 by continuous electron density map, which is critical for catalysis by PLP-dependent decarboxylase. Gel filtration showed that the active (pH 4.8) and inactive (pH 7.0) forms of GAD are all dimer. The residues (Ser126, Ser127, Cys168, Ile211, Ser276, His278 and Ser321) play important roles in anchoring PLP cofactor inside the active site and supporting its catalytic reactivity. The mutant T215A around the putative substrate pocket displayed an 1.6-fold improvement in catalytic efficiency (k_{cat}/K_m) compared to the wild-type enzyme (1.227 mM^{-1} S^{-1} versus 0.777 mM^{-1} S^{-1}), which was the highest activity among all variants tested. The flexible loop (Tyr308–Glu312), which is positioned near the substrate-binding site, is involved in the catalytic reaction, and the conserved residue Tyr308 plays a vital role in decarboxylation of L-Glu.

1. Introduction

As a unique pyridoxal phosphate enzyme, glutamate decarboxylase (GAD, EC 4.1.1.15) can catalyze the irreversible decarboxylation of L-glutamate (L-Glu) or its salts into γ-aminobutyrate (GABA) [1]. GABA, a major inhibitory neurotransmitter, has many physiological functions such as induction of hypotensive effects, treatment of epilepsy and strong secretagogue of insulin, and plays an important part in maintaining human health [2–4]. Therefore, GABA has been extensively applied for various functional foods and pharmaceuticals [5,6].

In contrast to chemical synthesis methods, enzymatic production of GABA catalyzed by GAD appears to be promising because of its cheap and easily obtainable substrate (L-Glu), excellent catalytic efficiency, simple reaction procedure and environmental friendliness [7,8]. GAD has been found in many microorganisms including *Escherchia coli* [9], lactic acid bacteria (LAB) [10,11], and *Monascus* species [12]. LAB possess special physiological activities and are categorized as “generally regarded as safe”, and have been applied for developing the health-promoting food and biologically active supplements [13]. Because of its safety and eco-friendliness, LAB fermentation may enable the production of naturally health-oriented foods enriched with GABA. The use of LAB as ideal sources of *gad* genes and cell factories for GABA production has been extensively investigated in the past few years [10,11,14]. Furthermore, GAD and GABA play important roles in acid or stress resistance in LAB [15,16]. Unfortunately, the GAD crystal structure of LAB remains unclear, and detailed investigations of the catalytic mechanism have been hampered.

Among the variety of reviewed LAB species, *Lactobacillus brevis* is the most frequently isolated species from traditional fermented

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foods with high GABA productivity [8,11,12,14]. In our previous study, a GABA-producing microorganism, *Lb. brevis* CGMCC 1306, was isolated from fresh milk and found to possess high GAD activity [7,8,17]. Yu et al. [18] reported that the C-terminal region of GAD from *Lb. brevis* CGMCC 1306 is crucial in the pH-activity relationship, and the last 14 C-terminal residues truncation extended its activity toward near-neutral pH. However, with the lack of GAD structural information, its modification for improving its activity or thermostability by rational or semirational ways is insufficient. Several structures of GAD have been determined to date. For example, the crystal structure and functional analysis of GAD from *E. coli* (PDB ID: 1XEY, 2DGK, 2DGL, 1PMM, 1PMO) and *Homo* sapien (2OKJ, 2OKK) have been reported [19–21]. To gain better insights into its catalytic mechanism and structure-function relationship, the crystallization and X-ray diffraction of GAD from *Lb. brevis* CGMCC 1306 were conducted in this work. In addition, structure-guided site-directed mutagenesis of GAD were conducted for residues around the active site and flexible loop with higher crystallographic B-factors (Y308-E312).

2. Materials and methods

2.1. Gene cloning, expression and protein purification

The gene encoding GAD (GenBank accession number, GU987102.1) from *Lb. brevis* CGMCC 1306 was sub-cloned into the pET-28a(+) expression vector by the restriction sites *Nde* I and *Hind* III to introduce an *N*-terminal His-Tag. The pET-28a(+)-GAD construct was confirmed by sequencing (GenScript Corp., Nanjing, China), and transformed into the host *E. coli* BL21 (DE3) competent cells by heat shock.

E. coli BL21 (DE3) carrying pET-28a(+)-GAD were cultivated in LB medium containing kanamycin (50 μg/mL) at 37 °C. When an optical density (OD_{600} nm) between 0.6 and 0.8 was obtained, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and the culture continued, with shaking at 150 rpm (25 °C, 18 h), to allow for protein expression. The cells were centrifuged at 6000×g for 8 min at 4 °C, and resuspended in lysis buffer (20 mM Tris-HCl buffer, 500 mM NaCl, 40 mM imidazole, 0.1 mM PLP, pH 7.8). Following cell lysis by ultrasonication at 4 °C in an ice-water bath, the cell extract was centrifuged at 12,000×g at 4 °C for 30 min. Recombinant GAD with one His_6-tag in its *N*-terminal was purified from the resulting clarified lysate by Ni-affinity chromatography. Purified proteins were eluted in elution buffer (20 mM Tris-HCl buffer, 500 mM NaCl, 400 mM imidazole, 0.1 mM PLP, pH 7.8), and concentrated using 10 kDa cut-off membrane (Millipore, Billerica, MA, USA). To further purify the protein for crystallization, the samples were digested by thrombin at 16 °C, and purified by gel filtration chromatography on a Sephadex G-200 column (GE Healthcare, NJ, USA) in buffer A (10 mM sodium phosphate buffer, NaCl 137 mmol/L, KCl 2.7 mmol/L, pH 7.4). The sample was concentrated to 10 mg/mL by ultrafiltration using 10 kDa cut-off membrane, and used for crystallization trials.

2.2. Crystallization of GAD

Purified GAD protein was crystallized at 14 °C using the hanging-drop vapor-diffusion method. After screening of the crystallization conditions, the best crystals appeared in the condition containing 0.2 M ammonium sulfate, 0.1 M sodium acetate, 10% v/v PEG 2000 as a precipitant, and 30% w/v dextran sulfate sodium salt (Mr 5000) as an additive. Diffraction data with 2.2 Å resolution were collected at 100 K on the beamline BL17U1 of the Shanghai Synchrotron Radiation Facility (China). The collected data sets were indexed, integrated and scaled using the HKL2000 software package [22]. The structures were solved by molecular replacement with the CCP4i program PHASER using the structure of *E. coli* (PDB code 1XEY) as the model [23]. The resulting model of native GAD showed a final crystallographic R_{work} of 18.76% and R_{free} of 23.08%. Model building was performed by COOT [24], and all of the model qualities were checked with PROCHECK. The crystallographic refinement and data collection statistics are summarized in Table 1.

2.3. Protein Data Bank accession code

Coordinates of the protein atoms together with structure factors have been deposited in the Protein Data Bank of Research Collaboration for Structural Bioinformatics (RCSB) with the following accession code: 5GP4 for GAD.

2.4. Activity assay

To determine the enzymatic activity, L-Glu decarboxylation reaction was carried out at 37 °C with 20 μg of enzyme in 200 μL of acetate buffer (pH 4.8, 100 mM) in the presence of 100 μM PLP and 100 mM L-Glu for 15 min. Enzyme reactions were terminated by boiling the assay mixture for 5 min. A high-performance liquid chromatography (HPLC) assay was used for detecting GABA concentration as described previously [7]. One unit of activity was equal to the amount of pure enzyme that produced 1 μmol GABA per min under the conditions described above. The specific activity is expressed as U/mg of protein. Steady-state kinetic measurements were performed with various L-Glu concentrations (0–100 mM). Plots of enzymatic activities vs. substrate concentrations were fit to a hyperbola function using non-linear regression to obtain the kinetic parameters of k_{cat} and K_m.

3. Results and discussion

3.1. The overall structure of GAD

The crystal structure of GAD in complex with PLP was determined by X-ray crystallography, and refined to a resolution of 2.2 Å with the space group $C222_1$ (Fig. 1). The data for crystallographic processing and structure refinement statistics are summarized in Table 1. As shown in Fig. 1A, the asymmetric unit contains three polypeptide chains which form an asymmetric trimer with 468 amino acid residues per subunit. The monomer A and B interface had 35 hydrogen bonds within 3.2 Å and 22 salt bridges, and the monomer A and C interface had 16 hydrogen bonds within 3.2 Å and 18 salt bridges according to analysis of protein interfaces using the PDBePISA server [25].

The recombinant GAD was eluted from the column as a single peak corresponding to the dimer (apparent molecular weight, 108 kDa) at pH 7.0, and the GAD at pH 4.8 was also eluted as a dimer (apparent molecular weight, 112 kDa) (Fig. S1). Gel filtration suggested that the observed trimer which only results from crystallographic packing was not the biological form, and the monomer A and monomer B tended to form the biological dimer (Fig. 1B and Fig. S2).

Although all 468 residues of GAD in this work were visible in the x-ray electron density maps, residues 1–13, 455–468 in each monomer were not modeled because of a poor electron density. The *N*-terminal domain (residues 14–57) of GAD is formed by one α-helix (residues 41–53). The PLP-binding domain (residues 58–364) contains the cofactor binding site and exhibits an α/β fold consisting of a central seven-stranded mixed β-sheet (residues 119–123, 157–163, 179–183, 204–212, 242–247, and 269–277 form parallel strands; residues 287–293 form an antiparallel strand) surrounded by seven α-helices (residues 92–110, 125–148,

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