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Structural insights into domain movement and cofactor specificity of glutamate dehydrogenase from *Corynebacterium glutamicum*

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ABSTRACT

Glutamate dehydrogenase (GDH) is an enzyme involved in the synthesis of amino acids by converting glutamate to α-ketoglutarate, and vice versa. To investigate the molecular mechanism of GDH, we determined a crystal structure of the *Corynebacterium glutamicum*-derived GDH (*Cg*GDH) in complex with its NADP cofactor and α-ketoglutarate substrate. *Cg*GDH functions as a hexamer, and each *Cg*GDH monomer comprises 2 separate domains; a Rossmann fold cofactor-binding domain and a substrate-binding domain. The structural comparison between the apo- and cofactor/substrate-binding forms revealed that the *Cg*GDH enzyme undergoes a domain movement during catalysis. In the apo-form, *Cg*GDH exists as an open state, and upon binding of the substrate and cofactor the protein undergoes a conformation change to a closed state. Our structural study also revealed that *Cg*GDH has cofactor specificity for NADP, but not NAD, and this was confirmed by GDH activity measurements. Residues involved in the stabilization of the NADP cofactor and the α-ketoglutarate substrate were identified, and their roles in substrate/cofactor binding were confirmed by site-directed mutagenesis experiments.

1. Introduction

Synthesis of amino acids is a vital process in almost all organisms, and nitrogen metabolism is a key reaction in this process. Glutamate dehydrogenases (GDHs) are involved in the tricarboxylic acid cycle and convert L-glutamate to α-ketoglutarate (2-oxoglutarate) through the oxidative deamination reaction. The reductive amination reaction using α-ketoglutarate provides nitrogen for several other biosynthetic pathways [1].

Based on their cofactor specificity, GDH enzymes can be categorized into 3 sub-families; NAD(H)-dependent (EC 1.4.1.2), NADP(H)-dependent (EC 1.4.1.4), and NAD(H)/NADP(H) dual-specific GDHs (EC 1.4.1.3) [2]. NAD(H)-dependent GDHs are commonly involved in glutamate catabolism, whereas NADP(H)-dependent GDHs are required for ammonium assimilation [3]. The dual-specific mammalian GDHs can utilize both NAD(H) and NADP(H) cofactors with comparable efficacy, and are known to be allosterically controlled by small molecules [3–5].

GDHs have been isolated and sequenced from various organisms and can be divided into 2 different types based on their oligomeric states. NADP(H)-specific bacterial and fungal GDHs and NAD(H)/NADP(H) dual-specific vertebrate GDHs function in hexameric form, with a molecular weight per subunit of approximately 50 kDa. NAD(H)-dependent GDHs have either a hexameric structure with around 48 kDa molecular weight, or have a tetrameric structure with around 115 kDa molecular weight [4,6–8]. Generally, in hexameric GDHs from bacteria, each subunit divides into 2 domains, with a deep cleft between each domain. The N-terminal substrate-binding domain is involved in hexamer formation and the C-terminal cofactor (NAD(H)/NADP(H)) binding domain exhibits a modified Rossmann fold [6].

In the present study, we report a crystal structure of GDH from *Corynebacterium glutamicum* (*Cg*GDH) in an apo-form and in complex with its cofactor and substrate. Based on the structural and biochemical studies of *Cg*GDH, we also reveal that the enzyme undergoes an open/closed conformational change upon binding of its cofactor and substrate. Using site-directed mutagenesis experiments, we also identified residues involved in the stabilization of the NADP cofactor and α-ketoglutarate substrate.

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2. Materials & methods

2.1. Cloning, expression and purification

Cloning, expression, purification, and crystallization of *Cg*GDH will be described in detail elsewhere (Son et al., in preparation). Briefly, the *Cg*GDH coding gene was amplified by polymerase chain reaction (PCR) using genomic DNA from *C. glutamicum* strain ATCC 13032 as a template. The PCR product was then subcloned into pProEX-HTa (Invitrogen) resulting in a 6x-histag followed by a TEV protease (rTEV) cleavage site at the N-terminus. The resulting expression vector pProEX-HTa:*Cg*GDH was transformed into an *Escherichia coli* BL21(DE3)-T1^{R} strain, which was grown in 1 L of LB medium containing 100 mM ampicillin at 37 °C. At an OD_{600} of 0.6, expression of *Cg*GDH protein was induced by adding 1 mM IPTG, and the culture medium was maintained for a further 20 h at 18 °C. The culture was harvested by centrifugation at 4000 × *g* for 20 min at 4 °C. The resulting cell pellet was resuspended in buffer A (40 mM Tris–HCl, pH 8.0) and disrupted by ultrasonication. The cell debris was removed by centrifugation at 13,500 g for 25 min and the lysate was applied to an Ni-NTA agarose column (Qiagen). After washing with buffer A containing 30 mM imidazole, the bound proteins were eluted with 300 mM imidazole in buffer A. The 6x-histag was removed by treatment with rTEV. Finally, trace amounts of contaminants were removed by size-exclusion chromatography using a Superdex 200 prep-grade column (320 ml, GE Healthcare) equilibrated with buffer A. All purification experiments were performed at 4 °C.

2.2. Crystallization, data collection, and structure determination

Crystallization of the purified *Cg*GDH protein was initially performed with commercially available sparse-matrix screens, including Index, PEG ion I and II (Hampton Research), Wizard Classic I and II, Wizard CRYO I and II (Rigaku) and Structure Screen I and II (Molecular Dimensions), all using the sitting-drop vapor-diffusion method at 20 °C. Each experiment consisted of mixing 1.0 μl protein solution (140 mg/ml in 40 mM Tris–HCl, pH 8.0) with 1.0 μl reservoir solution and then equilibrating against 50 μl reservoir solution. The *Cg*GDH crystals of the best quality appeared in 17% polyethylene glycol 3350, 7% Tacsimate, pH 5.0 and 10 mM spermidine. The crystals were finished out with a loop larger than the crystals and flash-frozen by immersion in liquid nitrogen. Data were collected to a resolution of 2.3 Å at 7A beamline of the Pohang Accelerator Laboratory (PAL, Pohang, Korea), using a Quantum 270 CCD detector (ADSC, USA). All data were indexed, integrated, and scaled together using the HKL2000 software package [9]. The crystals of *Cg*GDH belonged to the space group $P2_1$. Assuming 12 *Cg*GDH molecules in an asymmetric unit, the crystal volume per unit of protein mass was 2.42 $Å^3$ Da^{-1}, which means the solvent content was approximately 49.16% [10].

The structure was determined by molecular replacement with the CCP4 version of MOLREP [11] using the structure of GDH from *Clostridium symbiosum* (PDB code 1BGV) as a search model. Model building was performed manually using the program WinCoot [12], and refinement was performed with CCP4 refmac5 [13] and CNS [14]. The data statistics are summarized in Table 1. The refined *Cg*GDH model will be deposited in the protein data bank.

2.3. Site-directed mutagenesis and activity assay

Site-specific mutations were created with the Quick Change kit (Stratagene), and sequencing was performed to confirm correct incorporation of the mutations. Mutant proteins were purified in the same manner as for wild type. For GDH activity assay, oxidation of NADPH to NADP was measured by monitoring the decrease of absorbance at 340 nm (extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}). All assays were performed with reaction mixture of 1 ml total volume, and performed at 30 °C. The reaction mixture contained 100 mM Tris–HCl, pH 8.0, 250 μM NADPH or NADH, 20 mM ammonium chloride, and 10 mM α-ketoglutarate. The reaction was initiated by the addition of enzyme to a final concentration of 40.8 nM.

Table 1
Data collection and refinement statistics.

	*Cg*GDH
Data collection	
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	171.22, 93.032, 187.88
α, β, γ (°)	90.00, 108.16, 90.00
Resolution (Å)	50.00–2.30 (2.34–2.30)*
R_{sym} or R_{merge}	11.4 (29.9)
$I/\sigma(I)$	21.14 (5.36)
Completeness (%)	98.6 (95.8)
Redundancy	5.6 (4.2)
Refinement	
Resolution (Å)	178.51–2.30
No. reflections	1,364,094
R_{work}/R_{free}	16.8/22.6
No. atoms	42,473
Protein	40,318
Ligand/ion	522
Water	1633
B-factors	25.184
Protein	24.161
Ligand/ion	26.157
Water	26.466
R.m.s. deviations	
Bond lengths (Å)	0.0151
Bond angles (°)	1.8203

*Number of xtals for each structure should be noted in footnote. *Values in parentheses are for highest-resolution shell.
[AU: Equations defining various *R*-values are standard and hence are no longer defined in the footnotes.].
[AU: Ramachandran statistics should be in Methods section at the end of Refinement subsection.].
[AU: Wavelength of data collection, temperature and beamline should all be in Methods section.].

3. Results and discussion

3.1. Overall structure of CgGDH

In order to elucidate the cofactor specificity, oligomeric status, and reaction mechanism of glutamate dehydrogenase from *C. glutamicum* (*Cg*GDH), we determined a crystal structure of the protein at 2.3 Å. The asymmetric unit of the crystal contained 12 molecules, corresponding to 2 hexameric structures of the protein. The atomic structure was in good agreement with the X-ray crystallographic statistics for bond angles, bond lengths, and other geometric parameters (Table 1). The *Cg*GDH monomer consists of 2 core domains: a substrate-binding domain (Met1-Leu205, Gly368-Ser393 and Tyr425-Ile447) and a cofactor-binding domain (Val206-Pro367 and Phe394-Asp424) (Fig. 1). The substrate-binding domain consists of 10 α-helices (α1-α8, α15 and α17) and 4 β-strands (β1-β4). The 4 β-strands form a β-sheet packed in the middle of the substrate-binding domain and the 10 α-helices are located on both sides of the β-sheet. The cofactor-binding domain consists of 7 α-helices (α9-α14 and α-16) and 6 β-strands (β5-β10) and forms an NAD(H) and/or NADP(H)-binding Rossmann fold conformation. A classical Rossmann fold has a $\beta_1\alpha_1\beta_2\alpha_2\beta_3$-motif, with a second nucleotide-binding motif ($\beta_4\alpha_4\beta_5\alpha_5\beta_6$). The 2 motifs form a parallel