Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray crystallographic studies of glutamate racemase from *Lactobacillus fermenti*

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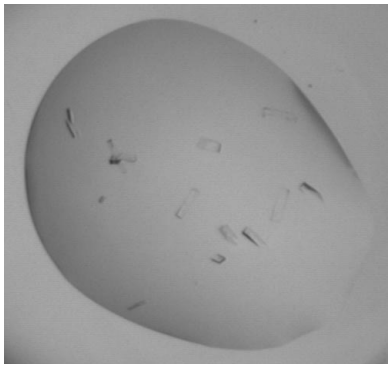
Received 7 October 2004
Accepted 29 December 2004
Online 20 January 2005



Glutamate racemase catalyzes the conversion of L-glutamic acid to D-glutamic acid and *vice versa*. Since D-glutamic acid is one of the essential amino acids present in peptidoglycan, glutamate racemase has been considered to be an attractive target for the design of new antibacterial drugs. Glutamate racemase from *Lactobacillus fermenti* has been crystallized by the hanging-drop vapour-diffusion method using polyethylene glycol 8000 as a precipitant. The crystals belong to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 98.32$, $b = 184.09$, $c = 45.99$ Å. The asymmetric unit contains one molecule, corresponding to a V_M value of 1.84 Å^3 Da^{-1}. A complete data set has been collected from the native enzyme at 2.28 Å resolution using a synchrotron-radiation source.

1. Introduction

The L-enantiomers of amino acids are predominant in living organisms. However, D-amino acids are also distributed in the biology of both prokaryotes and eukaryotes. These D-amino acids play crucial roles in rigidifying bacterial cell walls. D-Glutamic acid is one of the essential amino acids since it is required in the formation of peptidoglycan, which protects bacteria from osmotic lysis (Walsh, 1989).

Glutamate racemase (EC 5.1.1.3) catalyzes the interconversion of L-glutamic acid to D-glutamic acid and *vice versa*. D-Glutamic acid is an important source of bacterial cell-wall peptidoglycan and is produced by glutamate racemase in most Gram-negative and Gram-positive bacterial strains. It is clear that disruption of peptidoglycan biosynthesis is lethal to bacteria and glutamate racemase has therefore been considered to be an attractive target for the design of new antibacterial drugs. Glutamate racemase belongs to a class of cofactor-independent enzymes that includes aspartate racemase (Liu *et al.*, 2002) and diaminopimelate epimerase (Cirilli *et al.*, 1998). These enzymes employ a 'two-base' mechanism to catalyze racemization which utilizes a pair of cysteine residues as the catalytic acid and base (Gallo & Knowles, 1993; Koo & Blanchard, 1999; Yamauchi *et al.*, 1992). The glutamate racemase from *Lactobacillus fermenti* consists of 268 amino-acid residues with a calculated molecular weight of 28 300 Da. Studies on *L. fermenti* glutamate racemase using solvent-derived deuterium incorporation and mutagenesis have revealed that two cysteine residues play key roles in the deprotonation and protonation of the C^{α} atom of the substrate (Tanner *et al.*, 1993; Glavas & Tanner, 1999, 2001; Tanner, 2002).

Only one crystal structure of glutamate racemase has been reported to date. The crystal structure of glutamate racemase from *Aquifex pyrophilus*, a hyperthermophilic Gram-negative bacterium, has been solved (Hwang *et al.*, 1999). However, no strong interactions were observed between the D-glutamine present as a substrate-analogue inhibitor and the other active-site residues (Hwang *et al.*, 1999; Liu *et al.*, 2002). Therefore, more detailed investigations are required in order to elucidate the molecular mechanism of amino-acid racemization based on the two-base mechanism. The glutamate racemase from *L. fermenti* shows approximately 32% sequence identity to *A. pyrophilus* glutamate racemase. The crystal structure of *L. fermenti* glutamate racemase should provide the first three-dimensional structure of a cofactor-independent racemase from a Gram-positive bacterium and reveal the structural differences

between the enzymes from Gram-positive and Gram-negative bacteria. As the first step toward its structure elucidation, we have overexpressed glutamate racemase from *L. fermenti* and crystallized it. The crystallization conditions and preliminary X-ray crystallographic data are reported here.

2. Material and methods

2.1. Protein preparation

The transformants of *Escherichia coli* DH5α (pKG3) were grown in Luria–Bertani (LB) media supplemented with ampicillin (100 µg ml^{-1}). The glutamate racemase was purified on the basis of the method of Gallo & Knowles (1993). The pelleted cells were suspended in buffer *A* (30 m*M* Tris–HCl pH 7.5, 2 m*M* DTT, 1 m*M* PMSF). The cell lysate was prepared by ultrasonication in buffer *A* and clarified by centrifugation. The supernatant was loaded onto a column of DEAE-Sepharose (Sigma) that had been pre-equilibrated with buffer *A*. The column was washed with buffer *A* and then eluted with buffer *A* containing 200 m*M* NaCl. The eluant was concentrated using a Vivaspin concentrator (10 kDa molecular-weight cutoff membranes). The concentrated protein solution was exchanged with buffer *B* (20 m*M* Tris–HCl pH 7.8, 2 m*M* DTT) by repeated dilution and concentration using a Vivaspin concentrator. Portions of this protein solution were applied onto and eluted from a Q-Sepharose anion-exchange column (Sigma) that had been pre-equilibrated with buffer *B*. After each application of protein, the column was washed with buffer *B* and the column was eluted with a linear gradient of 0–300 m*M* NaCl in the same buffer, with a flow rate of 1 ml min^{-1}. Fractions containing racemase activity from the multiple runs were pooled and concentrated using a Vivaspin concentrator. The buffer of the concentrated protein solution was exchanged for buffer *C* (20 m*M* Tris–HCl pH 7.0, 2 m*M* DTT) as described above. The protein solution was then injected onto and eluted from a Q-Sepharose ion-exchange column in multiple runs. After the protein had been loaded, the column was washed with buffer *C* and then eluted with a linear gradient of 0–150 m*M* NaCl in buffer *C*. During the enzyme purification, the racemase activity was measured using a coupled enzyme-assay method as described by Gallo & Knowles (1993). The protein concentration was determined by using Bradford's method with bovine serum albumin as the standard. The absorbance at 280 nm was used to monitor the protein in the column effluent. Purification was monitored by gel electrophoresis. SDS–PAGE under denaturing conditions was carried out in 12%(*w*/*v*) polyacrylamide gels using Precision Protein Standard (Bio-Rad) as a reference protein for molecular-weight estimation. The protein bands were detected by staining with Coomassie Brilliant Blue R. The fractions containing racemase activity were pooled and concentrated to 10 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.0, 2 m*M* DTT and stored at 203 K. The final purified glutamate racemase was at least 95% pure as judged on a polyacrylamide gel.

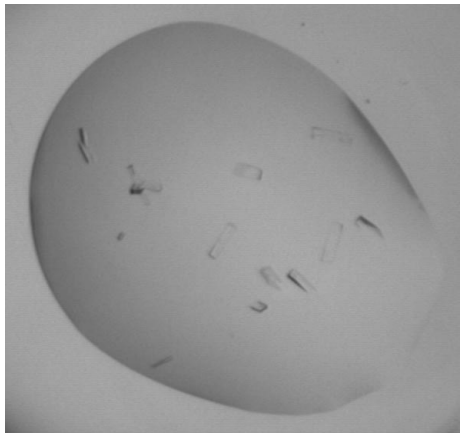


Figure 1
Crystals of glutamate racemase from *L. fermenti*. A crystal of dimensions 0.10 × 0.10 × 0.20 mm was used for X-ray diffraction data collection.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$C222_1$
Resolution (Å)	30–2.28 (2.37–2.28)
No. unique observations	17873 (1917)
Completeness (%)	90.9 (89.9)
R_{sym}†	0.148 (0.286)
$I/\sigma(I)$	6.3 (3.6)

† $R_{sym} = \sum |I - \langle I \rangle| / \sum \langle I \rangle$.

2.2. Crystallization and data collection

The initial crystallization screening was performed using Hampton Crystal Screen I and II kits by the hanging-drop vapour-diffusion method at room temperature by mixing 2 µl protein solution (10 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.0 with 2 m*M* DTT) with 2 µl reservoir solution (Jancarik & Kim, 1991). Each hanging drop was placed over 0.5 ml reservoir solution. Initial crystals were obtained using two solutions (Nos. 40 and 42) from Hampton Crystal Screen I. Crystal Screen I condition No. 40 [20%(*v*/*v*) 2-propanol, 0.1 *M* sodium citrate pH 5.6, 20%(*w*/*v*) PEG 4K] produced several thin plate-shaped crystals and Crystal Screen I condition No. 42 [20%(*w*/*v*) PEG 8K, 0.05 *M* potassium phosphate] produced similar shaped or a few rod-shaped crystals. The thin plate-shaped crystals from Crystal Screen I condition Nos. 40 and 42 were unstable at room temperature and diffracted poorly. However, the rod-shaped crystals, which were smaller in size (0.1 × 0.1 × 0.2 mm), diffracted to higher resolution and grew within 5 d.

The crystal was transferred into cryoprotection solution containing 22%(*v*/*v*) glycerol, 20%(*w*/*v*) PEG 8K, 0.05 *M* potassium phosphate, scooped up in a cryoloop and frozen in liquid nitrogen. It was then mounted on the goniometer in a stream of cold nitrogen at 100 K. X-ray diffraction data were collected from the cooled crystal using a Bruker Proteum 300 CCD at beamline 6B at Pohang Light Source (PLS), South Korea. The crystal-to-detector distance was set to 230 mm. A total of 150 images were collected with a 1° oscillation range per image. The wavelength of the synchrotron X-rays was 1.12714 Å and a 0.1 mm collimator was used. The crystal was rotated through a total of 150°, with 1.0° oscillation per frame. Diffraction data were collected to 2.28 Å resolution, integrated and scaled with the *DENZO* and *SCALEPACK* crystallographic data-reduction package (Otwinowski & Minor, 1997).

3. Results and discussion

The crystals suitable for X-ray analysis were obtained from the optimized crystallization conditions (Fig. 1) and diffracted to at least 2.2 Å. The autoindexing procedure performed with *DENZO* indicated that the crystals belong to an orthorhombic space group, with unit-cell parameters $a = 98.32$, $b = 184.09$, $c = 45.99$ Å, $\alpha = \beta = \gamma = 90°$. The space group was determined to be $C222_1$ on the basis of systematic absences. A total of 162 082 measured reflections were

merged into 17 873 unique reflections with an R_{merge} (on intensity) of 14.8%. The merged data set is 90.9% complete to 2.28 Å. Assuming the presence of one molecule per asymmetric unit, the calculated Matthews coefficient (V_M) value was 1.84 Å^3 Da^{-1} (Matthews, 1968). The solvent content of the crystal was calculated to be 33.1%. Data-collection statistics are given in Table 1.

Molecular replacement was attempted using the 2.3 Å structure of *A. pyrophilus* glutamate racemase (PDB code 1b73; Hwang *et al.*, 1999) as a search model. However, none of our attempts provided a clear solution. Therefore, a crystal of the selenomethionine-substituted protein has been obtained under the same conditions in order to solve the phase problem. The structure will be determined using the MAD method (Hendrickson *et al.*, 1990) and the structural details will be described in a separate paper.

The authors are most grateful to Dr Martin E. Tanner at British Columbia University for providing the cloned glutamate racemase (pKG3). We thank Dr H. S. Lee and his staff for assistance during data collection at beamline BL-6B of Pohang Light Source, South Korea. Experiments at PLS were supported in part by the Functional Proteomics Center, 21C Frontier Program of the Korea Ministry of Science and Technology. This work was supported by a Korea University Grant.

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