Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of glutamate racemase from *Aquifex pyrophilus*, a hyperthermophilic bacterium

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Received 7 October 1998
Accepted 11 January 1999

Glutamate racemase catalyzes the reversible reaction of L-glutamate to D-glutamate, an essential component of the bacterial cell wall. Glutamate racemase from *Aquifex pyrophilus* has been crystallized by the hanging-drop vapor-diffusion method using polyethylene glycol 6000 as a precipitant. The crystals belong to space group $P6_122$ or $P6_522$ with unit-cell parameters $a = b = 72.1$, $c = 185.02$ Å. The asymmetric unit contains one molecule, corresponding to a V_m value of 2.35 Å^3 Da^{-1}. Complete data sets from a native and a mercury-derivative crystal have been collected at 2.0 and 2.3 Å resolution, respectively, using a synchrotron-radiation source.

1. Introduction

The bacterial cell wall contains several kinds of D-amino acids as components of peptidoglycan, and these D-amino-acid residues are thought to protect the cell walls from proteolytic digestion. D-Glutamate is incorporated into peptidoglycan through its addition to UDP-*N*-acetylmuramyl-L-alanine, a peptidoglycan precursor, and this reaction is catalyzed by UDP-*N*-acetylmuramoylalanine-D-glutamate ligase (E.C. 6.3.2.9; Mengin-Lecreulx *et al.*, 1989). D-Glutamate can be synthesized from D-alanine and α-ketoglutarate by D-amino-acid aminotransferase (E.C. 2.6.1.21; Tanizawa *et al.*, 1989) or alternatively from L-glutamate by glutamate racemase (E.C. 5.1.1.3; Nakajima *et al.*, 1986). Glutamate racemase has been found only in bacteria, including the pathogenic *Helicobactor pylori* and *Mycobacterium tuberculosis*. Therefore, the enzyme can be considered as a potential target for novel antibacterial drugs.

Racemases can be grouped into two classes depending on their requirement for cofactors: alanine and arginine racemase require pyridoxal 5′-phosphate (PLP; Walsh, 1989) which forms a Schiff base with the substrate amino acid, whereas aspartate (Yamauchi *et al.*, 1992; Yohda *et al.*, 1996) and glutamate racemase (Gallo *et al.*, 1993; Yagasaki *et al.*, 1995) are cofactor-independent enzymes. Glutamate racemase has been isolated from at least eight different bacteria including *Escherichia coli*. We have recently cloned, purified and characterized glutamate racemase from *Aqueifex pyrophilus*, a hyperthermophilic bacterium which grows optimally at 358 K (Kim & Yu, unpublished data). Glutamate racemase from *A. pyrophilus* shares 26–37% amino-acid sequence identity with other glutamate racemases. Biochemical studies of glutamate racemase from *Lactobacillus fermenti* 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). These two cysteines and surrounding residues are strictly conserved among all glutamate racemases (Gallo *et al.*, 1993). Despite their biological importance, no structural information is available on any cofactor-independent amino-acid racemases. It is only recently that the crystal structures of PLP-dependent alanine racemase (Shaw *et al.*, 1997; Stamper *et al.*, 1998) and metal-dependent mandelate racemase (Neidhart *et al.*, 1991; Schafer *et al.*, 1996), which do not show any sequence homology to glutamate racemase, have been determined.

Crystallographic studies of glutamate racemase from *A. pyrophilus* would provide the first three-dimensional structure of cofactor-independent racemase, which in turn would reveal the structural basis for the racemization mechanism of D- or L-glutamate. Furthermore, the crystal structure of glutamate racemase should provide insights into new antibiotics. Here, we report the crystallization and preliminary crystallographic studies of glutamate racemase from *A. pyrophilus*.

2. Results and discussion

Details of the cloning, purification and characterization of glutamate racemase from *A. pyrophilus* will be published elsewhere. Briefly, glutamate racemase was overexpressed in *E. coli* and purified by three steps: heat treatment at 358 K for 1 h and CM-Sepharose ion-exchange chromatography, followed by gel-filtration chromatography. The enzyme was

Table 1
Data-collection statistics for native and mercury-derivative crystals.

X-ray source	Synchrotron		Rigaku RU-200	
Data set	Native	EMTS	Native	EMTS
Wavelength (Å)	1.000	1.000	1.541	1.541
Resolution (Å)	2.0	2.7	3.0	3.5
Total observations	176663	71818	31309	12584
Unique observations	19997	13375	6221	3953
Completeness (%)	99.2	88.0	99.2	97.4
$R_{\rm merge}$†	0.116	0.082	0.106	0.124

† $R_{\rm merge} = \sum_i \sum_j (I_{ij} - _j) / \sum_i I_{ij}$.

concentrated to 10 mg ml^{-1} in a 50 m*M* Tris–HCl buffer (pH 7.0) and stored at 203 K.

Crystallization was performed by the hanging-drop vapour-diffusion method. Initial screening trials used the sparse-matrix method (Jancarik & Kim, 1991) at 291 K. Rod-shaped or thin plate crystals appeared within 2 d from several drops containing 6–12%(*w*/*v*) PEG 4K, 6K or 8K. These crystals were unstable at room temperature and diffracted poorly. However, a few hexagonal-shaped crystals were formed from drops containing equal volumes of the protein solution (10 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.0) and 6%(*w*/*v*) PEG 6K. While diffraction from these crystals was observed to 3.5 Å resolution using a rotating-anode generator, the crystals were not reproducible. To reproduce these crystals with improved diffraction quality, we used microseeding techniques. Seeds were prepared by mechanical agitiation of an original hexagonal bypyramidal shaped crystal using a homogenizer. Microseeds produced by this method were found to be useful for up to three months. Seeds were added to drops containing 2 µl of glutamate racemase (10 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.0) solution and 2 µl of reservoir solution [6%(*w*/*v*) PEG 4K, 2 m*M* DTT, 0.1 *M* sodium citrate, pH 7.0] and equlibrated with 1 ml of reservoir solution. Crystals of typical dimensions 0.15 × 0.1 × 1.2 mm were grown consistently by this method. Although crystals were stable for at least six months at room temperature, they showed high sensitivity to X-rays. To overcome this problem, the crystals were slowly transferred to a cryo-protection solution containing 30% glycerol, 12%(*w*/*v*) PEG 6K and 100 m*M* sodium citrate (pH 6.5) and were flash frozen at 83 K in a stream of cold nitrogen.

Initial data were collected from a MAR Research 30 image-plate detector mounted on a Rigaku RU-200 rotating-anode generator; the crystal diffracted to 3.0 Å resolution (Table 1). Higher resolution data (to 2.0 Å) have been obtained at 83 K on an ADSC 4 panel CCD area detector using synchrotron radiation at beamline 5.0.2 of the Advanced Light Source (LBNL, Berkeley). All X-ray data were processed using the *DENZO*/*SCALEPACK* crystallographic data-reduction package (Otwinowski & Minor, 1997). The space group of the crystals was determined to be $P6_122$ or $P6_522$, based on the symmetry of the diffraction patterns and the systematic absences along 00*l* characteristic of the sixfold screw axis. The post-refined parameters of the unit cell were $a = b = 72.1$, $c = 185.02$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The calculated molecular weight of the protein is 27993 Da per subunit; assuming one monomer per asymmetric unit yields a Matthews coefficient of 2.48 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 45.4%.

It has been shown that two other racemases, alanine racemase and mandelate racemase, consist of a domain with an α/β barrel fold in addition to other domains. Structure-prediction studies (Hwang & Cho, unpublished data) using *VADAR* (written by D. S. Wishart) suggest that *A. pyrophilus* glutamate racemase may also fold into an α/β-barrel structure. Therefore, we performed molecular replacement using several different models with α/β-barrels as search models. However, none of our attempts provided a clear solution.

We have therefore initiated a search for heavy-atom derivatives. A mercury-derivative crystal was prepared by soaking the crystals with 2 m*M* ethyl mercury thiosalicylate (EMTS) for 2 d. This crystal was isomorphous to the native crystal and the data set scaled to the native data set with an $R_{\rm iso}$ of 0.189. An isomorphous difference Patterson map calculated using the program *PHASES* (Furey & Swaminathan, 1997) showed several strong peaks in Harker sections. The peaks could be interpreted in terms of a single heavy-atom site (0.53, 0.73, 0.01). Parameters of the heavy-atom site were refined using the program *PHASES* to give a phasing power of 1.2 at 3.0 Å resolution. Model building based on the electron-density map calculated from the phases obtained using the mercury derivative and screening of other heavy-atom derivatives are now in progress.

We thank Dr Tom Earnest and Dr Li-Wei Huang at the Advenced Light Source (LBNL, Berkeley) for help with data collection. This work was supported by KIST 2000 program and Biotech 2000 program from MOST.

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