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Expression, purification and preliminary X-ray analysis of crystals of *Bacillus subtilis* glutamate racemase

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Glutamate racemase (MurI, RacE; E.C.5.1.1.3) catalyses the cofactor-independent conversion of L-glutamate to D-glutamate, an essential step in the synthesis of components of the bacterial cell wall. The gene for RacE from *Bacillus subtilis* has been cloned and the protein expressed in *Escherichia coli*, purified and crystallized in the presence of L-glutamate using the hanging-drop method of vapour diffusion with diammonium tartrate as the precipitant. The crystals belong to the monoclinic space group $C2$, with approximate unit-cell parameters $a = 133.6$, $b = 60.1$, $c = 126.2$ Å, $\beta = 117.6^\circ$. Consideration of the possible values of V_M suggests that the asymmetric unit contains either two ($V_M = 3.75$ Å^3 Da^{-1}) or three ($V_M = 2.5$ Å^3 Da^{-1}) subunits. The crystals diffract X-rays to at least 2.1 Å resolution on a synchrotron-radiation source and are suitable for structural studies. Determination of the structure may provide insight into the molecular basis of substrate recognition and catalysis by this enzyme.

1. Introduction

The last 20 years have seen an alarming increase in bacterial resistance to existing antibiotics (Levy, 1998), but a decline in the introduction of new antibacterial agents. The threat posed to public health by this situation has led to an increase in the search for new antibiotics utilizing the data provided by genome studies. As a contribution to this area, we have been engaged in the structural analysis of the protein products derived from the set of essential bacterial genes from *Bacillus subtilis* (Kobayashi *et al.*, 2003) in order to provide a toolkit for the discovery of novel anti-infective agents.

Defects or disruption of the cell wall of bacteria leads to cell lysis and death owing to the high internal osmotic pressure of the cell. The biosynthesis of bacterial cell-wall components is therefore an essential pathway for many pathogenic bacteria (Cox *et al.*, 2000) and has long been accepted as a target for antibiotic action (Bugg & Walsh, 1992; Chu *et al.*, 1996; de Dios *et al.*, 2002). Thus, penicillins, cephalosporins and glycopeptide drugs such as vancomycin all act by inhibiting key steps in the assembly of the peptidoglycan layer of bacterial cell walls (Neu, 1992). The biosynthetic pathways that lead to peptidoglycan production provide a wide range of targets for drug delivery, which include enzymes involved in non-ribosomal peptide-bond synthesis and in the synthesis and the utilization of D-amino acids, which form components of the cell wall.

D-Glutamate is an important biosynthetic building block involved in the formation of the peptidoglycan layer and can be produced from L-glutamate by the action of glutamate racemase (RacE; Glavas & Tanner, 2001). This step has been shown to be essential for the viability of *Streptococcus pneumoniae* (Baltz *et al.*, 1999), *Escherichia coli* (Doublet *et al.*, 1992, 1993), *Staphylococcus haemolyticus* (Pucci *et al.*, 1995) and *B. subtilis* (Kobayashi *et al.*, 2003), suggesting that RacE forms a possible target for the development of antibacterial agents. Recently, the first potent inhibitors of RacE showing whole-cell antibacterial activity have been described (de Dios *et al.*, 2002).

RacE is a member of a small group of amino-acid racemases and epimerases that operate in a cofactor-independent fashion and that includes proline racemase, aspartate racemase and diaminopimelate epimerase (Glavas & Tanner, 2001). The *Aquifex pyrophilus* and *E. coli* enzymes have been reported to be dimeric (Hwang *et al.*, 1999; Yoshimura *et al.*, 1993), whilst a monomeric state has been proposed for RacE from *Lactobacillus* (Gallo & Knowles, 1993). Biochemical studies have suggested that the catalysis by glutamate racemase uses a 'two-base' mechanism involving deprotonation of the substrate at the α-position to form an anionic intermediate followed by reprotonation in the opposite stereochemical sense (Glavas & Tanner, 2001). These studies, together with the recent structure determination of RacE from *A. pyrophilus* (Hwang *et al.*, 1999), have revealed that two conserved cysteines act as the bases during the enzyme's catalytic cycle (Gallo *et al.*, 1993). Sequence comparisons with aspartate racemase suggest that the latter enzyme operates

via a similar mechanism (Yamauchi *et al.*, 1992).

The structure determination of *A. pyrophilus* RacE and the analysis of crystals soaked in solutions containing a weak inhibitor, D-glutamine, provided the first structural details of the architecture of the active site of this enzyme (Hwang *et al.*, 1999). This analysis suggested that the mode of glutamine inhibition involves the positioning of this substrate analogue in the active site in a reverse orientation to that of glutamate, with the amide group of the glutamine side chain occupying the position in the active site of the planar intermediate formed at the C^{α} atom of glutamate following the initial deprotonation step of the enzyme's catalytic cycle. Thus, glutamate binding to RacE is thought to occur in an orientation that swaps the position of the C^{α} atom and its associated amino and carbonyl groups and the side chain of the amide group of glutamine (Hwang *et al.*, 1999). However, the direct binding of glutamate has not as yet been observed and further structural data are required to confirm the proposed roles of the residues that lie in the active-site pocket. The sequence of the *A. pyrophilus* enzyme is 38% identical to that of *B. subtilis* RacE and includes the conservation of all key residues. In this paper, we describe the cloning, purification, crystallization and preliminary X-ray analysis of *B. subtilis* RacE as a contribution to understanding the wider structure–activity relationships of this enzyme.

2. Materials and methods

Degenerate oligonucleotide primers corresponding to the *RacE* gene sequence obtained from the SubtiList website (Moszer *et al.*, 1995; Moszer, 1998) and custom-synthesized using an Applied Biosystems 394 DNA/RNA Synthesiser, in both orientations, were used to amplify a *RacE* gene-specific product from genomic DNA isolated from a culture of *B. subtilis* 168 strain using DNeasy Tissue Kit (Qiagen). The PCR products were ligated into a pETBlue-1 vector using an Acceptor Vector Cloning kit (Novagen), creating an expression vector, pMAT1. The PCR products were sequenced using an ABI PRISM 377 DNA Sequencer to verify that no mutation had been introduced. An *E. coli* cell line [Tuner (DE3) pLacI] was transformed with pMAT1 and this strain was grown in LB medium at 310 K with vigorous aeration until the OD_{590} reached 0.6, after which expression was induced with 1 m*M* IPTG. The cells were grown for a further 4 h in order to achieve maximal yield of RacE and then harvested by centrifugation at 4000*g* for 20 min at 277 K and the cell paste stored at 253 K. To obtain selenomethionine-labelled protein, RacE was overexpressed using a modification of the protocol described above as reported in Mehanni *et al.* (2002).

The purification of RacE followed an equivalent protocol for both the methionine and selenomethionine-labelled protein. Cell paste from a 0.5 l growth (approximately 2 g cell paste) was suspended in ten volumes of buffer *A* (50 m*M* Tris–HCl pH 8.0) and disrupted by ultrasonication for 3 × 20 s at 16 µm amplitude. Cell debris was removed by centrifugation at 43 000*g* for 15 min. The supernatant fraction was loaded onto a column packed with 35 ml DEAE-Sepharose Fast Flow (Amersham Biosciences) and the proteins were eluted with a 300 ml NaCl concentration gradient from 0 to 0.5 *M* in buffer *A*. Fractions eluting at approximately 0.15 *M* NaCl contained the highest protein concentration [estimated by the method of Bradford (1976) using the Bio-Rad Protein Assay reagent] and were combined; 3 ml of 4 *M* ammonium sulfate was added per 4 ml protein solution to give a final ammonium sulfate concentration of 1.9 *M*. The precipitated proteins were removed by centrifugation at 43 000*g* for 5 min. The supernatant fraction was then applied onto a 20 ml column packed with Phenyl-Toyopearl 650S (ToyoSoda, Japan) and equilibrated with 1 *M* ammonium sulfate in buffer *A*. The protein was eluted from this column with a 200 ml reverse gradient of ammonium sulfate concentration from 1 to 0 *M* in buffer *A*. Fractions eluting at approximately 0.75 *M* ammonium sulfate contained the highest protein concentration and were combined. Volume was reduced to 1–1.5 ml using a VivaSpin concentrator with molecular-weight cutoff 10 kDa (Viva-Science). The sample was then loaded onto a gel-filtration column (Hi-Load Superdex-200; Amersham Biosciences) equilibrated with 0.1 *M* NaCl in buffer *A*. Gel filtration was performed with the same buffer, 2 ml fractions were collected and protein-peak fractions were combined. The protein was concentrated on a VivaSpin concentrator (10 kDa MWCO) to 0.5 ml, washed with ten volumes of buffer *B* (10 m*M* Tris–HCl pH 8.0) and then concentrated to 10 mg ml^{-1}.

Activity assays were performed in buffer *C* (50 m*M* sodium phosphate buffer pH 8.0) with 1 m*M* L-glutamate at 303 K, using a RacE concentration of approximately 0.4 µ*M*. Ellipticity at 204 nm was monitored using a Jasco J-810 CD Spectropolarimeter (Jasco). The molar ellipticity of glutamic acid was taken as 31.0 mdeg cm^{-1} m*M*$^{-1}$.

Gel-filtration analysis was performed using a Superdex-200 10/300 GL column on a ÄKTA Purifier (Amersham Biosciences) at a flow rate of 0.5 ml min^{-1}, calibrated by running a set of protein standards in buffer *C*. The protein standards, their molecular weights and volumes of elution were as follows: thyroglobulin, 669 kDa (9.25 ml); apoferritin, 443 kDa (10.55 ml); β-amylase, 200 kDa (11.75 ml); alcohol dehydrogenase, 150 kDa (12.60 ml); bovine albumin, 66 kDa (13.45 ml); egg albumin, 45 kDa (14.06 ml); carbonic anhydrase, 29 kDa (15.80 ml); trypsin inhibitor, 20.1 kDa (15.95 ml); cytochrome *c*, 12.3 kDa (17.30 ml); aprotinin, 6.5 kDa (19.40 ml). The column void volume (V_0) was estimated as 8.0 ml. 0.1 ml samples of RacE protein at a concentration of 3 mg ml^{-1} in the presence of a number of additives, including L- and D-glutamate, L- and D-glutamine and L-aspartate, in buffer *C* were applied onto the gel-filtration column, which had been equilibrated with buffer *C* in the presence of the corresponding additive. The volume of the peak elution (V_e) was determined and the apparent molecular weight of RacE was then estimated by comparison of its relative mobility (V_e/V_0) to a plot of log MW *versus* V_e/V_0 determined from the standards.

Crystallization trials were attempted using Crystal Screens I and II and the PEG/Ion screen (Hampton Research). Crystals were grown using the standard hanging-drop vapour-diffusion technique by mixing 2 µl protein solution at a concentration of 10 mg ml^{-1} in the presence of 15 m*M* L-glutamate (sodium salt, pH 7.5) with an equal volume of precipitant and then equilibrating against the same precipitant at 290 K.

Data collection at cryogenic temperatures utilized crystals stabilized in a solution containing 20%(*w*/*v*) PEG 3350, 0.2 *M* diammonium tartrate, 15 m*M* L-glutamate and 20%(*w*/*v*) glycerol as a cryoprotectant. Crystals were flash-frozen in a stream of nitrogen gas at 100 K using an Oxford Cryosystems Cryostream device. Multiple-wavelength anomalous diffraction (MAD) data were collected on beamline ID14-4 equipped with an ADSC Quantum 4 detector at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Three wavelengths were chosen near the selenium-absorption edge based on an X-ray fluorescence scan of the frozen crystal in order to maximize the f'' component (λ_1, peak), to minimize the f' component (λ_2, inflection) and to maximize $\Delta f'$ (λ_3,

remote). The data for each wavelength were processed individually and scaled in such a way as to preserve the anomalous signal using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997) and subsequently handled by the *CCP4* package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The overexpression of RacE in *E. coli* resulted in high-level expression of the protein, all of which was found to reside in the soluble fraction. RacE elutes from the gel-filtration column with an approximate molecular weight of 37 kDa, consistent with a monomeric structure. The purity of RacE after the final step varied slightly from preparation to preparation and was estimated by SDS–PAGE to be in range 85–95% (Fig. 1). The typical yield was approximately 10 and 5 mg of RacE protein per gram of cell paste for the methionine- and selenomethionine-labelled proteins, respectively. The specific activity of the purified enzyme was in the range 12–19 U mg^{-1}. It is tenfold lower than that of the *Lactobacillus* enzyme (Gallo & Knowles, 1993), but twice as high as that of the *E. coli* enzyme (Doublet *et al.*, 1993) and about tenfold higher than *A. pyrophilus* (Kim *et al.*, 1999). The k_{cat} value for the conversion of L-glutamate to D-glutamate for RacE was determined to be 6.8 s^{-1}.

RacE from *A. pyrophilus* (Hwang *et al.*, 1999) and *E. coli* (Yoshimura *et al.*, 1993) have been reported to be dimeric, whilst a monomer has been proposed for the *Lactobacillus* enzyme (Gallo & Knowles, 1993). Gel-filtration studies on *B. subtilis* RacE suggest that the quaternary structure of this enzyme is in equilibrium between a monomer and a dimer, shifting to a monomeric form for the free enzyme and to a dimer in the presence of either L- or D-glutamate or glutamine (Table 1). In contrast, in the presence of aspartate or higher salt concentrations the enzyme remains monomeric.

Table 1
Data for gel-filtration analysis of RacE under different conditions.

Buffer composition	No. experiments	Volume of peak elution (V_e) (ml)	V_e/V_0	Apparent MW (Da)
50 m*M* sodium phosphate pH 8.0 (buffer *C*)	14	15.00 ± 0.2	1.875 ± 0.025	37700 ± 4000
10 m*M* L-Glu in buffer *C*	3	14.06 ± 0.03	1.755 ± 0.004	60250 ± 1200
10 m*M* D-Glu in buffer *C*	1	14.10	1.763	58900
10 m*M* L-Gln in buffer *C*	1	14.40	1.800	49000
100 m*M* L-Gln in buffer *C*	2	14.32 ± 0.08	1.790 ± 0.010	51300 ± 2300
10 m*M* D-Gln in buffer *C*	2	14.29 ± 0.04	1.786 ± 0.005	53700 ± 1300
100 m*M* D-Gln in buffer *C*	1	14.06	1.756	60100
10 m*M* Asp in buffer *C*	2	15.11 ± 0.02	1.889 ± 0.002	34700 ± 700
500 m*M* sodium phosphate pH 8.0	2	15.30 ± 0.02	1.913 ± 0.003	30200 ± 700

Table 2
X-ray data collection for RacE crystallized in the presence of L-glutamate.

Values in parentheses are for the highest resolution shell.

Data set	Peak (λ_1)	Inflection (λ_2)	Remote (λ_3)
Wavelength (Å)	0.97570	0.9796	0.95380
Resolution (Å)	15–2.1 (2.15–2.1)	15–2.4 (2.46–2.4)	15–2.1 (2.15–2.1)
Observed reflections	166772	120576	164777
Unique reflections	50425	34740	50734
Completeness (%)	96.5 (73.5)	99.7 (98.2)	97.1 (78.8)
R_{merge}† (%)	7.5 (29.5)	8.0 (30.7)	7.7 (31.4)
$\langle I/\sigma(I)\rangle$	14.4 (3.0)	13.8 (2.8)	13.4 (3.0)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average.

Initial crystals of RacE in the presence of L-glutamate were obtained using the PEG/Ion crystallization kit (Hampton Research) condition No. 38 [20%(*w*/*v*) PEG 3350, 0.2 *M* diammonium tartrate]. After optimization of the crystallization conditions, plate-like crystals with maximum dimensions of 0.15 × 0.1 × 0.1 mm were grown from 16%(*w*/*v*) PEG 3350, 0.2 *M* diammonium tartrate in 48 h.

A single selenomethionine-derivative crystal was used for the MAD data collection, which was performed to a maximum resolution of 2.1 Å. Analysis of the preliminary data set with the autoindexing routine in *DENZO* (Otwinowski & Minor, 1997) is consistent with the monoclinic space group *C*2, with unit-cell parameters a = 133.6, b = 60.1, c = 126.2 Å, β = 117.6°. Details of the data-collection statistics are presented in Table 2. Taking the subunit molecular weight to be 30 kDa, this would suggest that the crystals contain either two or three subunits in the asymmetric unit, with corresponding V_M values of 3.75 or 2.5 Å^3 Da^{-1}, respec-

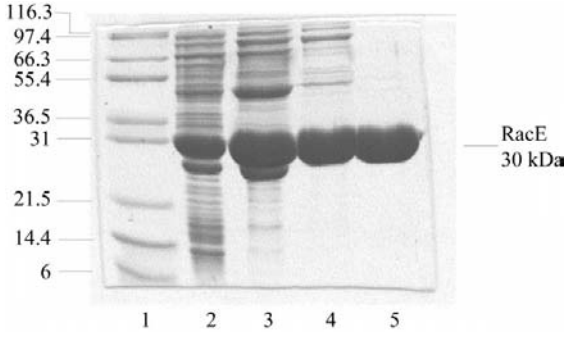

Figure 1
12% SDS–PAGE showing the purification of RacE. Lane 1, Mark12 MW protein markers (kDa) (Invitrogen); lane 2, crude extract; lane 3, combined fractions after DEAE anion-exchange chromatography; lane 4, combined fractions after hydrophobic chromatography; lane 5, RacE protein collected after gel-filtration chromatography.

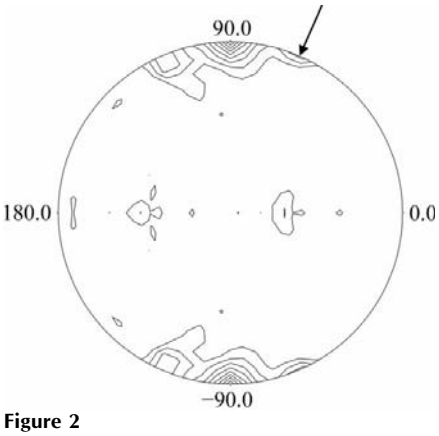

Figure 2
A stereographic projection of the κ = 180° section of a self-rotation function calculated using data from the RacE–glutamate crystals over the resolution range 15–3.0 Å with an integration radius of 15 Å. The tilt of the rotation axis away from the crystallographic c axis (ω, sometimes also called ψ) increases radially from 0 or 180° at the centre to 90° at the perimeter. The peak at ω = 90° and φ = 68° (arrowed) is a possible non-crystallographic twofold axis and represents 54% of the origin.

tively, both of which lie within the range given by Matthews (1977). A self-rotation function was calculated using the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) using data in the resolution range 15–3 Å and a 15 Å radius of integration (Fig. 2). A significant peak equal to 54% of the origin was found on the $\kappa = 180^\circ$ section at $\omega = 90^\circ$ and $\varphi = 68^\circ$, implying that the cell contains a non-crystallographic twofold axis lying in the *ab* plane.

A full structure determination of these crystals of *B. subtilis* RacE is currently under way. Once completed, it is hoped that the structure will provide new information on the location of the substrate-binding site, thereby contributing to the development of novel antibacterial agents targeted against the enzyme.

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