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Crystallization and preliminary X-ray crystallographic studies of salt-tolerant glutaminase from *Micrococcus luteus* K-3

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Glutaminase from the marine bacterium *Micrococcus luteus* K-3 (*Micrococcus* glutaminase) is a salt-tolerant protein which shows equivalent activities both in the absence and the presence of 3 *M* sodium chloride and is distinct from halophilic proteins, which are inactivated in the absence of salt. To investigate the mechanisms of the salt-tolerant adaptation of *Micrococcus* glutaminase, the glutaminase and its major fragment containing about 80% of the protein were crystallized using the hanging-drop vapour-diffusion method. The glutaminase crystals belong to space group *P*622, with unit-cell parameters $a = b = 111.4$, $c = 210.9$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$, and diffract to 2.6 Å resolution. The fragment crystals belong to space group *F*222, with unit-cell parameters $a = 115.7$, $b = 116.4$, $c = 144.9$ Å, $\alpha = \beta = \gamma = 90^\circ$, and diffract to 2.4 Å resolution. Data from selenomethionine (SeMet) substituted glutaminase crystals and from SeMet-substituted fragment crystals were collected to 2.6 and 2.4 Å resolution, respectively. Structural analyses of the glutaminase and its fragment are currently being attempted using the multiwavelength anomalous diffraction (MAD) phasing method.

1. Introduction

Glutaminase (EC 3.5.1.2), which catalyzes the hydrolytic degradation of L-glutamine to L-glutamic acid, plays an important role in the food-processing industry, since it increases the amount of the savoury flavouring L-glutamic acid. However, glutaminases from the *koji* mould (*Aspergillus oryzae*), which is commonly used for soy-sauce fermentation, are markedly inhibited by the high salt concentrations in the soy-sauce fermentation process (about 3 *M* sodium chloride; Yano *et al.*, 1988). Therefore, the salt-tolerant glutaminase from *M. luteus* (Moriguchi *et al.*, 1994) may play a potentially significant role in industrial processes that require high-salt environments.

Recently, there has been considerable interest in the structures of proteins from microbes which occur in extreme environments. Three crystal structures of halophilic proteins, dihydrofolate reductase from *Haloferax volcanii* (Pieper *et al.*, 1998) and ferredoxin and malate dehydrogenase from *Haloarcula marsimortui* (Frolow *et al.*, 1996; Richard *et al.*, 2000), have highlighted a variety of interesting protein/solvent features involved in halophilic adaptation. *Micrococcus* glutaminase is a salt-tolerant protein which is active both in the absence and in the presence of 3 *M* sodium chloride (Moriguchi *et al.*, 1994) and is distinct from halophilic proteins, which are inactivated in the absence of salt. No crystal structure of a salt-tolerant protein has so far been solved.

The three-dimensional structures of glutaminase–asparaginases from two bacteria, *Pseudomonas* 7A (36.2 kDa; Lubkowski *et al.*, 1994) and *Acinetobacter glutaminasificans* (35.5 kDa; Ammon *et al.*, 1988) have been determined. However, *Micrococcus* glutaminase shows only 11.9 and 12.4% sequence identity to glutaminase–asparaginases from *Pseudomonas* and *Acinetobacter*, respectively. The low sequence identities between *Micrococcus* glutaminase and the structurally characterized glutaminase–asparaginases could be indicative of a unique structure of *Micrococcus* glutaminase.

Micrococcus glutaminase (48.3 kDa) is much larger than other bacterial glutaminases (Wakayama *et al.*, 1996) such as the 33.0 kDa glutaminase from *Rhizobium etli* (Calderon *et al.*, 1999), the putative 36.2 kDa glutaminase from *Escherichia coli* (Yokotsuka *et al.*, 1987) and the putative 36.2 kDa glutaminase from *Bacillus subtilis* (Shimizu *et al.*, 1991). *Micrococcus* glutaminase appears to be divided into two domains by sequence-comparison analysis: the conserved N-terminal domain (amino-acid residues 1–300; 31.6 kDa) and the unique C-terminal domain (amino-acid residues 301–456; 16.7 kDa). The N-terminal domain shares 54.7 and 35.4% identity in primary structure with glutaminase–asparaginase from *Coryne-*

bacterium glutamicum (30.6 kDa; Pompejus *et al.*, 2001) and *B. halodurans* (33.5 kDa; Takami *et al.*, 2000), respectively. *Micrococcus* glutaminase tends to degrade, forming a large C-terminally truncated fragment with an apparent molecular mass of 38.5 kDa, after incubation at 277 K for a month, possibly owing to a minute amount of contaminating proteases (unpublished data).

To understand the salt-tolerant adaptation of the *Micrococcus* glutaminase at a molecular level, we attempted to determine the tertiary structure of the *Micrococcus* glutaminase and its fragment. In this paper, we report the crystallization and preliminary X-ray analysis of the *Micrococcus* glutaminase and its fragment.

2. Materials and methods

2.1. Protein preparation

E. coli JM 109 cells transformed with pKSGHE 3-1, a high-expression plasmid containing the *Micrococcus* glutaminase gene (Nandakumar *et al.*, 1999), were cultured at 303 K for 18 h. IPTG was added to the culture medium to a final concentration of 0.1 m*M* 2 h prior to harvesting. The buffer used (buffer I) was 10 m*M* Tris–HCl buffer pH 7.2 containing 10 m*M* $MgCl_2$ with 10% glycerol. The cells (0.1 g ml^{-1}) were disrupted by sonication in buffer I and the cell debris was removed by centrifugation. The supernatant was dialysed against buffer I and then applied to a DEAE-Toyopearl column (TOSOH) equilibrated with buffer I. The column was washed with buffer I containing 130 m*M* NaCl and the enzyme was then eluted using buffer I containing 150 m*M* NaCl. The fractions containing glutaminase activity were pooled and dialysed against buffer I. The enzyme purity was examined using SDS–PAGE. The recombinant protein was purified to homogeneity with a yield of 0.4 mg per gram of cells and had a higher specific activity (1965 U mg^{-1}) than the protein preparation from the original strain (1230 U mg^{-1}; Nandakumar *et al.*, 1999). In labelling the glutaminase with SeMet, the *E. coli* met^- auxotrophic strain B834 (DE3) (Novagen) was used as a host for plasmid transformation and cells were grown at 310 K in M9 media including 25 mg l^{-1} SeMet and with addition of IPTG (to a final concentration of 0.1 m*M*). The SeMet-substituted glutaminase was purified by the same procedure as for the wild-type protein. Incubation of native and SeMet-substituted glutaminase at high concentration (10 mg ml^{-1}) at 277 K for at least one month produced the 38.5 kDa fragment for both species.

2.2. Crystallization and data collection

The glutaminase was concentrated to 10 mg ml^{-1} in 10 m*M* Tris buffer pH 7.2 containing 10 m*M* $MgCl_2$ and 10%(*v*/*v*) glycerol. Crystallization conditions were screened using Crystal Screen I (Hampton Research, USA) with the microbatch method. 2 µl of the protein solution was mixed in a 1:1 ratio with each of the crystallization solutions and the resultant 4 µl drops were incubated at 293 K. Hexagonal plate crystals (Fig. 1) were found in a drop containing 0.05 *M* HEPES buffer pH 7.5, 0.7 *M* trisodium citrate dihydrate (Hampton Crystal Screen condition I-38) in 1 d. Subsequent refinement of the conditions gave the best crystallization conditions, which employ the hanging-drop method and use 0.75 ml reservoir solution consisting of 0.1 *M* HEPES buffer pH 7.5, 0.84 *M* sodium citrate and 6 µl of protein solution consisting of 5 mg ml^{-1} protein, 0.05 *M* HEPES buffer pH 7.5, 0.7 *M* sodium citrate, 5 m*M* $MgCl_2$, 5% glycerol. After treatment with Paratone oil, the crystals were flash-cooled in a gaseous nitrogen flow at 100 K. In the Paratone oil treatment, a crystal from the crystallization drop was transferred into the oil and then moved around to remove excess surrounding mother liquor using a cryoloop large enough to accommodate it.

The SeMet-substituted glutaminase was crystallized under the same conditions as those optimized for the native glutaminase. Although the crystal-growth period and the crystal morphology were identical to those of the native glutaminase, the SeMet-substituted glutaminase crystals were slightly less reproducible and were inconsistent in size. The glutaminase fragment (about 38.5 kDa) crystallized under identical conditions to those of the intact glutaminase. The fragment crystals were trapezoidal plates (Fig. 2), distinct in shape from the hexagonal plates of the glutaminase crystals. The SeMet-substituted glutaminase fragment also formed trapezoid plate crystals under conditions identical to those for the native glutaminase.

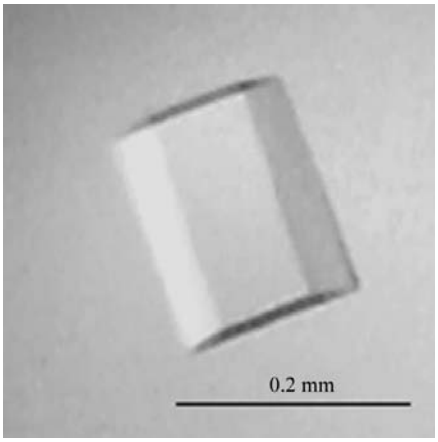

Figure 1
Micrograph of a hexagonal plate crystal. The dimensions of the crystal are approximately 0.2 × 0.14 × 0.14 mm.

3. Results and discussion

The asymmetric unit of the glutaminase crystals contains two glutaminase molecules. For the SeMet-substituted glutaminase crystals, three wavelengths, 0.9791 Å (peak), 0.9793 Å (edge) and 0.9700 Å (remote), were selected based on an X-ray absorption spectrum at the Se *K* edge. The CCD detector was placed at a distance of 200 mm and the exposure time ranged from 5 to 7 s for different crystals, allowing us to collect a 180° data set (1° frames) in a few hours. The three-wavelength MAD data set from the SeMet-substituted glutaminase crystal was collected at beamline BL41XU, SPring8, Japan. The diffraction data were integrated using *MOSFLM* (Leslie, 1992) and scaled using *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Data statistics are summarized in Table 1.

The diffraction quality of the fragment crystals was better than that of the glutaminase crystals under the conditions optimized for the glutaminase crystals. The asymmetric unit contains one glutaminase fragment. For the SeMet-substituted fragment crystals, three wavelengths, 0.9791 Å (peak), 0.9793 Å (edge) and 0.9700 Å (remote), were selected based on an X-ray absorption spectrum at the Se *K* edge. Data

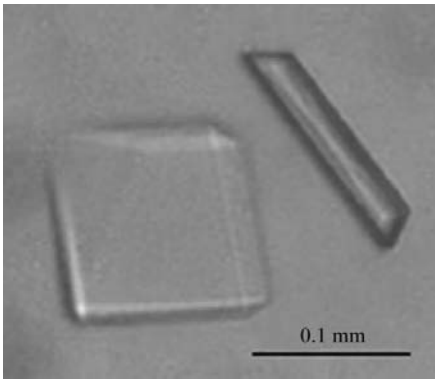

Figure 2
Micrograph of trapezoidal plate crystals. The dimensions of the crystals are approximately 0.13 × 0.13 × 0.02 mm.

Table 1
Data-collection statistics.

	Intact glutaminase	Glutaminase fragment
Unit-cell parameters (Å, °)	$a = b = 111.4$, $c = 210.9$, $\alpha = \beta = 90$ $\gamma = 120$	$a = 115.7$, $b = 116.4$, $c = 144.9$, $\alpha = \beta = \gamma = 90$
Space group	*P*222	*F*622
Molecular weight (kDa)	48.3	38.5
No. of molecules per AU	2	1
Wavelength (Å)		
Peak	0.9791	0.9791
Edge	0.9793	0.9793
Remote	0.9700	0.9700
Resolution range (Å)	2.6	2.4
No. of unique reflections	40208	16718
Completeness (%)	94.4	93.4
$R_{\rm merge}$ (%)	11.8	6.5
Redundancy	3.5	6.8

statistics are summarized in Table 1. MAD analyses using the data sets from the SeMet-substituted glutaminase crystals and the SeMet-substituted fragment crystals are in progress.

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