Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of the *Escherichia coli* UDP-MurNAc-tripeptide D-alanyl-D-alanine-adding enzyme (MurF)

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Received 17 May 1999
Accepted 14 September 1999

Crystals of the *Escherichia coli* UDP-MurNAc-tripeptide D-Ala-D-Ala-adding protein (MurF), which catalyzes the formation of the last metabolite of the bacterial cell-wall building block, have been grown in hanging-drop vapor-diffusion trials using PEG 8K as a precipitating agent. The crystals belong to hexagonal space group $P6_1$ or $P6_5$, with unit-cell dimensions $a = b = 74$, $c = 425$ Å. The asymmetric unit contains two molecules, with a crystal volume per protein mass (V_m) of 3.4 Å^3 Da^{-1} and a solvent content of about 64% by volume. A native data set to 2.8 Å resolution has been obtained from a frozen crystal using a synchrotron X-ray source.

1. Introduction

The bacterial cell wall is constructed by the cross-linked polymeric mesh peptidoglycan which surrounds and contains the plasma membrane and cytoplasm of the cell. The building blocks of the peptidoglycan are synthesized in the cytoplasm. Four ADP-forming ligases, MurC, MurD, MurE and MurF, catalyze the assembly of its peptide moiety by successively adding L-alanine, D-glutamate, a diaminoacid and D-alanyl-D-alanine to the UDP-*N*-acetylmuramic acid. The D-Ala-D-Ala-adding enzyme (MurF) catalyzes the synthesis of the final cytoplasmic peptidoglycan precursor UDP-MurNAc-pentapeptide (Bugg & Walsh, 1992).

Antibiotics which are available currently block either the step before the D-Ala-D-Ala-adding reaction (for example, D-cycloserine) or the step after that, which inhibits the cross-linking of the building blocks (for example, β-lactams, penicillin *etc.*). There is no drug available which targets the D-Ala-D-Ala-addition reaction.

Of the ADP-forming ligases which catalyze the assembly of the peptidoglycan peptide moiety, crystal structures of MurD and DD-ligase are available (Bertrand *et al.*, 1997; Fan *et al.*, 1994). An X-ray crystallographic analysis of MurF (the D-Ala-D-Ala-adding ligase), which is an attractive target for the development of new antibacterial agents, has been undertaken. This report describes the crystallization and preliminary X-ray analysis of native MurF.

2. Materials and methods

2.1. Expression and purification

The MurF gene was amplified from genomic DNA of *Escherichia coli* strain K37 using the following oligonucleotides as primers 5′-GACATATGATTAGCGTAACCCTTAGC-3′ and 5′-CTGTCGACCTAACATGTCCCATTCTCCT-3′. The PCR fragment was subcloned as an *Nde*I/*Sal*I fragment into pET30a(+) (Novagen) using standard molecular-biology techniques (Maniatis *et al.*, 1989) and the DNA sequence was confirmed to be identical to published data (EMBL access code X55034). *E. coli* strain TOP10F′ (Invitrogen) was used for plasmid construction and BL21(DE3) (Stratagene) for protein expression. Protein expression was performed essentially as described previously (Pryor & Leiting, 1997), using minimal medium with *cas*amino acids and exchanging the medium prior to inducing the cells with 0.5 m*M* IPTG (isopropyl-β-D-thiogalactopyranoside, Sigma) for 20 h at 295 K. After expression, the cells were collected by centrifugation (3400*g*, 277 K, 10 min), washed with ice-cold TNE (10 m*M* Tris–HCl pH 7.4, 100 m*M* NaCl, 10 m*M* EDTA) and either used directly for purifica-

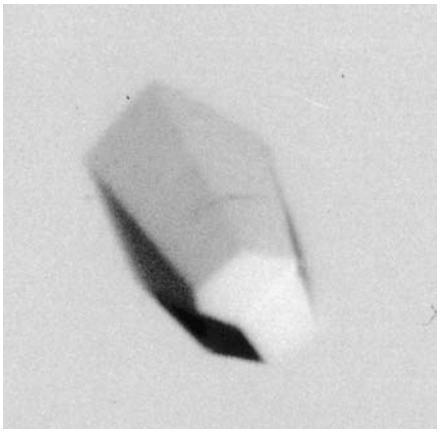

Figure 1
Single crystal of MurF with dimensions 0.2 × 0.2 × 0.8 mm.

Table 1
2.8 Å resolution data-collection statistics.

Resolution (Å)	R_{sym}†	I/σ	Completeness (%)
8.16	0.073	8.4	92
6.03	0.075	7.8	100
5.00	0.079	7.2	100
4.36	0.081	7.0	100
3.92	0.082	7.4	100
3.59	0.089	6.5	100
3.33	0.096	6.2	100
3.12	0.108	5.6	99
2.95	0.120	5.2	96
2.80	0.133	4.9	77
Overall	0.085		95

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

tion or first flash-frozen and stored in liquid nitrogen.

Cells from a 1 l culture were resuspended in 30 ml buffer *A* (10 m*M* Tris–HCl pH 7.4, 10 m*M* DTT, 1 m*M* EDTA) with 1 m*M* benzamidine (Sigma) and 1 m*M* pefabloc (serine protease inhibitor from Boehringer–Mannheim), lysed with a French press (20k cell from SLM Aminco, 8.3 MPa, single run at 277 K) and centrifuged (140 000*g*, 35 min, 277 K). The lysis supernatant was loaded onto a fresh Q-Sepharose column (Fast Flow resin, Pharmacia, 2.5 × 5 cm, flow rate 2.0 ml min^{-1}). The column was washed with at least 10 column volumes of buffer *A* and eluted with a 60 min gradient from 0 to 500 m*M* NaCl gradient in the same buffer. MurF-containing fractions were pooled, concentrated to >15 mg ml^{-1} (Biomax-10K, Millipore), divided into four parts and further purified by size-exclusion chromatography (Superdex 75, Pharmacia; column size 2.5 × 70 cm; buffer: 10 m*M* Tris–HCl pH 7.4, 150 m*M* NaCl, 10 m*M* DTT; maximum load 80 mg). MurF was finally purified on a phenyl Sepharose column after the addition of 1.2 *M* ammonium sulfate (Pharmacia; 2.5 × 20 cm; buffer: 10 m*M* Tris pH 7.4, 10 m*M* DTT, 1.2 *M* ammonium sulfate) and eluted with a 60 min inverse gradient from 1.2–0 *M* ammonium sulfate. The protein was flash-frozen in liquid nitrogen and stored at 203 K. Mass-spectral analysis revealed a molecular mass of 47 448.1 Da (calculated mass = 47 447.2 Da) and the enzyme activity was assayed as described previously (Anderson *et al.*, 1996).

3. Results and discussion

3.1. Crystallization

The purified protein was concentrated to 12.2 mg ml^{-1} in 10 m*M* Tris buffer at pH 7.4 in the presence of 10 m*M* DTT. Suitable crystallization conditions were screened by vapor diffusion using the hanging-drop method. The droplets contained an equal volume of the protein solution and the reservoir solution (5 µl each). Hexagonal rod-shaped crystals were obtained in a few days at 277 K using a reservoir solution containing 0.1 *M* Bis–Tris propane buffer at pH 9.4, 18% PEG 8K, 0.12 *M* $MgSO_4$ and 10% glycerol. The crystals grew to a maximum size of approximately 0.2 × 0.2 × 0.8 mm in about 20 d (Fig. 1).

3.2. X-ray diffraction

The crystals are temperature sensitive and were handled at 277 K. The X-ray diffraction data were collected at 100 K. The crystals were soaked in a cryoprotectant harvest solution consisting of reservoir solution with 20%(*v*/*v*) glycerol for 2 h at 277 K and were then cryo-cooled to 100 K using an MSC liquid-nitrogen cryosystem. The first native data set was collected on an R-AXIS IIC image plate using an MSC mirror monochromator system and Cu $K\alpha$ radiation from a Rigaku RU200 rotating-anode generator operated at 50 kV and 100 mA. The data were collected to 3.5 Å resolution as 1.5° oscillation frames and were processed and scaled using the *HKL* suite (Otwinowski & Minor, 1996). 90% of the possible data have been recorded with an R_{sym} of 9.0% [defined as $\sum(|I - \langle I\rangle|)/\sum I$] (15.0% in the highest resolution shell). The higher resolution data to 2.8 Å were collected at synchrotron beamline 17-ID of the Advanced Photon Source at Argonne National Laboratory. The wavelength was set to 1 Å. The data were collected as 0.25° oscillation frames and were processed with *DPS* software (Area Detector Systems Corporation) with a data completeness of 95%. The overall R_{sym} was 8.5% and R_{sym} for the highest resolution shell was 13.3%. Table 1 shows the detailed data statistics.

3.3. Space-group determination

The crystals belong to a hexagonal space group, with unit-cell dimensions $a = b = 74$, $c = 425$ Å, as determined from the diffraction data autoindexed with *DENZO* and refined with *SCALEPACK*. The X-ray diffraction patterns were inspected with the program *PHASES* (Furey & Swaminathan, 1997). Sixfold symmetry was found along the *c* axis. A mirror plane was found perpendicular to the *c* axis and there was no mirror plane parallel to it. The data revealed systematic absences along the *c** axis for reflections 00*l* with $l \neq 6n$. These symmetric patterns and systematic absences indicated that the crystals belong to the space group $P6_1$ or its enantiomorph $P6_5$. An assumption of two molecules per asymmetric unit led to an acceptable packing density V_m of 3.4 Å^3 Da^{-1}, corresponding to a solvent content of 64% (Matthews, 1968).

We thank Dr Lawrence C. Kuo for prompting this project, for his encouragement throughout this work and for his critical reading of the manuscript. We thank Dr Zhongguo Chen for his coordinative role within the crystallography group. We also thank Matt S. Anderson and Kenny K. Wong for testing enzyme activity, and James Cole for an analysis of the solution homogeneity of the protein. The 2.8 Å data were collected at beamline 17-ID in the facilities of the Industrial Macromolecular Crystallography Association Collaborative Access Team (IMCA-CAT) at the Advanced Photon Source. These facilities are supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with Illinois Institute of Technology (IIT), executed through the IIT's Center for Synchrotron Radiation Research and Instrumentation. Use of the Advanced Photon Source was supported by the US Department of Energy, Basic Energy Sciences, Office of Energy Research, under Contract No. W31-109-Eng-38. We would like to thank the staff at beamline 17-ID and Chris Nelson at Area Detector Systems Corporation for their help during data collection and data processing.

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