

Crystallization and preliminary X-ray analysis of UDP-*N*-acetylenolpyruvylglucosamine reductase (MurB) from *Staphylococcus aureus*

Melissa S. Harris,^{a,c,*} John T. Herberg,^{b,c} Joyce I. Cialdella,^{b,c} Joseph P. Martin Jr,^{b,c} Timothy E. Benson,^{a,c} Gil H. Choi^d and Eric T. Baldwin^{a,c}

^aStructural Analytical and Medicinal Chemistry, 301 Henrietta Street, Kalamazoo, MI 49007, USA, ^bProtein Science, 301 Henrietta Street, Kalamazoo, MI 49007, USA, ^cPharmacia, 301 Henrietta Street, Kalamazoo, MI 49007, USA, and ^dHuman Genome Sciences, Rockville, MD 20850-3338, USA

Correspondence e-mail:
melissa.s.harris@pharmacia.com

UDP-*N*-acetylenolpyruvylglucosamine reductase (MurB) is an essential enzyme in the bacterial cell-wall biosynthetic pathway, making it a potential therapeutic target for novel antibiotics. Diffraction-quality crystals of both the native and Se-methionine-expressed MurB from *Staphylococcus aureus* have been prepared by sitting-drop vapour diffusion from solutions containing polyethylene glycol (PEG) 8000, ammonium sulfate, sodium cacodylate pH 6.5 and dimethyl sulfoxide (DMSO). Crystals belong to the cubic space group $I2_13$, with unit-cell parameters $a = b = c = 178.99$ Å. X-ray data from these crystals were collected at the Advanced Photon Source 17-ID beamline and were used to solve the MurB structure to 2.3 Å resolution.

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1. Introduction

Antibiotics such as penicillin which target the biosynthesis of peptidoglycan in bacteria have been the mainstays for treatment of bacterial infections for decades. Recently, however, the emergence of antibiotic-resistant bacteria has increasingly become a major health threat. This unmet medical need has prompted a new interest in the development of potent orally available antibiotics. Among the many potential approaches to new antibiotics include those which target peptidoglycan biosynthesis but are not directed toward the same target as penicillin. UDP-*N*-acetylenolpyruvyl-glucosamine reductase (MurB) from *S. aureus* is such a target. Inhibition of this enzyme should result in disruption of the cell's ability to synthesize the disaccharide unit of the cell wall and should be bactericidal. Recently, others have described the crystallization and structure determination of the Gram-positive MurB enzyme from *Escherichia coli* (Benson *et al.*, 1995). We have crystallized the *S. aureus* enzyme as a first step in the structure-directed drug design of a MurB Gram-negative targeted antibiotic.

Bacterial peptidoglycan is a polymer of repeating disaccharide units which are cross-linked by a four- or five-residue amino-acid chain. One of the sugars, *N*-acetylmuramic acid, is produced by the action of the MurA and MurB enzymes. The enzyme MurA (UDP-*N*-acetylglucosamine enolpyruvyl transferase) condenses a molecule of UDP-*N*-acetylglucosamine with phosphoenolpyruvate to form enolpyruvyl-UDP-*N*-acetylglucosamine (EP-UDPGlcNAc) with the liberation of inorganic phosphate (Brown *et al.*, 1995;

Marquardt *et al.*, 1992). This enolpyruvyl product is subsequently reduced by UDP-*N*-acetylenolpyruvyl-glucosamine reductase (MurB) from the vinyl ether double bond to generate the *D*-lactyl ether (UDP-*N*-acetylmuramic acid). MurB has a bound flavin adenine dinucleotide (FAD) cofactor which is first reduced by NADPH and subsequently reduces the enolpyruvyl substrate (Benson *et al.*, 1993, 1994, 1995; Dhalla *et al.*, 1995). UDP-*N*-acetylmuramic acid is joined to L-Ala by MurC using ATP (Fig. 1).

We have crystallized *S. aureus* MurB and this report summarizes crystal optimization, cryoprotection and data collection. Success in this project required growing crystals from large drop volumes on sitting-drop bridges coated with silicone vacuum grease to eliminate firm attachment, growing crystals in mother liquor suitable for cryocooling and the diffraction characterization of a large number of crystals. Optimized crystals were used for the determination of the three-dimensional structure of *S. aureus* MurB (Benson *et al.*, 2001).

2. Materials and methods

2.1. Protein preparation

S. aureus MurB (36 kDa) cloned into pQE-10 (Qiagen) was obtained from Human Genome Sciences and expressed using UC 15169, *E. coli* construct K12S (F' *lacI^q*). Cultures in M9 media were induced with 1 mM isopropyl β -D-thiogalactopyranoside and reached maximal growth and expression ($A_{600} \approx 2.0$) after 4 h. Cells were harvested by centrifugation and frozen at 193 K. To enable a

Table 1
Data-collection statistics for MurB (edge, 0.97939 Å).

Resolution (Å)	No. of observations	Completeness (%)	Redundancy	$\langle I/\sigma \rangle$	R_{sym}^{\dagger}	R_{shell}
4.952	3918	89.20	6.46	41.7	0.052	0.052
3.931	7925	91.50	6.75	35	0.051	0.05
3.435	11959	92.60	6.93	19.6	0.058	0.075
3.121	16010	93.50	7.04	11.5	0.065	0.1
2.897	20114	94.20	7.12	7	0.071	0.132
2.726	24229	94.70	7.15	4.6	0.077	0.172
2.59	28347	95.20	7.12	3.2	0.082	0.221
2.477	32483	95.60	6.96	2.6	0.087	0.255
2.382	36418	95.50	6.79	1.8	0.091	0.316
2.3	40336	95.30	6.63	1.6	0.095	0.338

$$\dagger R_{\text{sym}} = \frac{\sum_{hkl} \sum_{i=1}^N |I_i^{hkl}| - I_i^{hkl}}{\sum_{hkl} \sum_{i=1}^N I_i^{hkl}}$$

structure determination by multiple anomalous dispersion (MAD) phasing, selenomethionine was incorporated into MurB through down-regulation of methionine biosynthesis just prior to induction of MurB (Van Duyne *et al.*, 1993; Benson *et al.*, 1995). The mass of the Se-Met-expressed MurB measured by electrospray mass spectrometry indicated full incorporation of the five selenomethionines into the protein.

Cell paste was resuspended using a Tekmar Tissuizer in buffer containing 50 mM Tris pH 7.8, 500 mM NaCl, 10% glycerol, 25 mM imidazole, 5 mM β -mercaptoethanol (BME) and 0.2 mg ml⁻¹ DNase. The suspension was homogenized using a Rannie homogenizer at 69 MPa and centrifuged at 39 200g for 60 min. The supernatant was filtered and applied to a nickel-charged Qiagen NTA Superflow column and washed with 50 mM Tris pH 7.8, 500 mM NaCl, 10% glycerol, 50 mM imidazole, 5 mM BME. Protein was eluted using the above buffer containing 300 mM imidazole at a flow rate of 60 ml h⁻¹. 10 mM dithiothreitol (DTT) was added immediately to the eluted material. Pooled MurB protein

was dialyzed for 22 h against nitrogen-sparged buffer (50 mM Tris pH 7.8, 500 mM NaCl, 10% glycerol, 10 mM DTT), sterile filtered, fractionated and stored in aliquots at 193 K. Protein concentration was 2.4 mg ml⁻¹ by amino-acid analysis.

Initial preparation of MurB for crystallization experiments revealed the protein to be unstable at 277 K and it could not tolerate successive freeze/thaw cycles. Freshly thawed protein was exchanged into buffer containing 20 mM HEPES pH 7.5, 5 mM BME or DTT and concentrated to 20 mg ml⁻¹ using Ultrafree 0.5 centrifugal filters with a Biomax 10K membrane (Millipore, Bedford, MA). Protein was used immediately for crystallization experiments. Overnight storage of protein prior to setup was detrimental to crystal formation and resulted in heavy precipitation in the crystallization drops.

2.2. Crystallization

MurB (20 mg ml⁻¹) was screened at 293 K against Hampton Crystal Screen and Crystal Screen 2 using hanging-drop vapour diffusion by mixing 1 μ l protein solution with 1 μ l reservoir solution (Jancarik & Kim, 1991; Cudney *et al.*, 1995). Hampton Crystal Screen condition 15 [30% PEG 8000, 0.1 M sodium cacodylate pH 6.5, 0.2 M (NH₄)₂SO₄] produced discrete pockets of yellow gelatinous precipitate. A systematic grid screen of the PEG 8000, ammonium sulfate concentrations and the pH resulted in tiny yellow plates after 4 d from drops containing 0.1 M sodium cacodylate pH 6.5, 15% PEG 8000, 0.5 M (NH₄)₂SO₄. Crystals were optimized by adjusting the PEG 8000

and (NH₄)₂SO₄ concentrations and by setting up larger drop sizes using sitting-drop vapour diffusion. Crystals large enough for X-ray diffraction analysis were obtained, but grew firmly attached to the bridge causing many crystals to fracture during harvesting. This problem was solved by coating the microbridge with a thin layer of silicone vacuum grease prior to setup. Crystals grown by this method no longer stuck to the bridge surface and could be easily scooped up for transfer into stabilization solution and/or cryosolution. Crystals were cryoprotected in 10% PEG 8000, 0.55 M (NH₄)₂SO₄, 0.1 M sodium cacodylate pH 6.5, 5 mM BME or DTT with 15% 2-methyl 2,4-pentanediol (MPD) and were cryocooled in liquid propane. Crystals diffracted to only 7 Å resolution on the home source and to 3.5 Å using synchrotron radiation. Several methods were attempted to improve diffraction quality of the crystals, including co-crystallization with substrates or additives. Screening with Hampton Additive Screen I produced crystals in most drops and optimization with a select set of additives did not show improvement in crystal size or diffraction quality (Michel, 1990; Cudney *et al.*, 1995; Sousa, 1995; Trakhanov & Quioco, 1995). Crystals grown with 10 mM EP-UDPGlcNAc increased growth in the thinnest crystal dimension and resulted in chunky hexagonal crystals; however, no improvement in diffraction was observed. Despite this negative result, 10 mM EP-UDPGlcNAc was included in subsequent experiments to achieve the desired crystal morphology.

Concurrent screening of MurB in the presence of potential inhibitors led to the discovery that supplemental DMSO during crystal growth significantly improved the diffraction quality of MurB crystals to 2.3 Å. DMSO was initially used to aid solubilization of the hydrophobic inhibitors during co-crystallization. The altered crystallization conditions were optimized using the purified MurB (Se-Met) at 20 mg ml⁻¹ in complex with 10 mM EP-UDPGlcNAc. Large single crystals grew in 1–2 weeks on grease-coated microbridges by mixing 5 μ l Se-Met MurB and 5 μ l reservoir solution (Fig. 2). Optimized crystallization conditions contained 0.1 M sodium cacodylate, 9.75% PEG 8000, 0.55 M (NH₄)₂SO₄, 20% DMSO and 5 mM BME and produced crystals of dimensions of up to 0.4 \times 0.4 \times 0.4 mm. The high DMSO concentration also proved to be an excellent cryoprotectant and

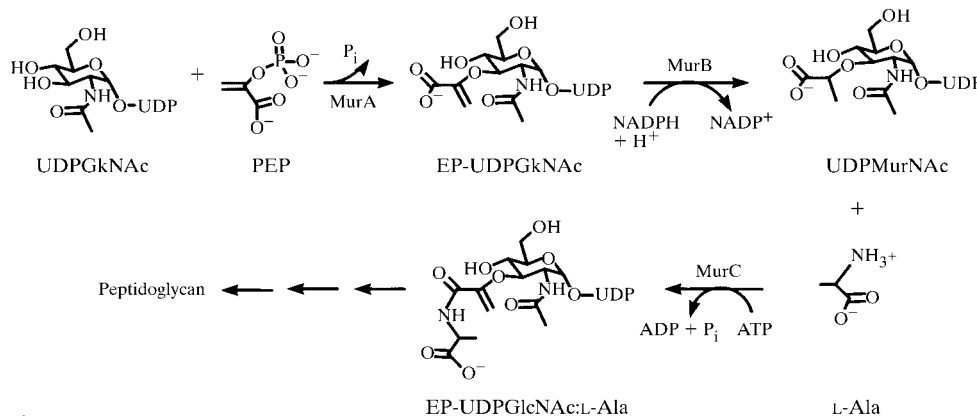


Figure 1
The biosynthetic context for MurB.

crystals could be removed from their drops and immediately cryocooled in liquid propane for low-temperature data collection.

2.3. Preliminary X-ray diffraction analysis

Data were collected using synchrotron radiation at the IMCA-CAT beamline 17-ID at the Advance Photon Source, Argonne National Laboratories. The unfocused monochromatic beam from the undulator had a σ_v of 6 μ rad and σ_H of 23 μ rad (approximate beamsize 1×3.5 mm). This beam passed through slits of 0.1×0.1 mm which restricted the beam to a 1.5 μ rad plane-wave. A collimator of 0.5 mm inside diameter was placed just before the crystal and acted as a scatter guard. At the beginning of the data collection, the ring current

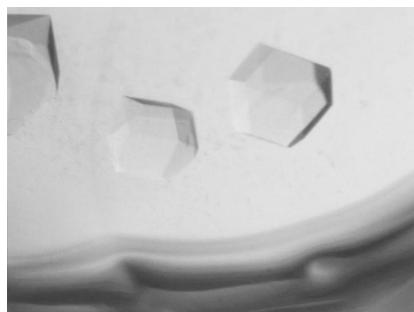


Figure 2
Crystals of MurB enzyme, which appear yellow owing to the presence of oxidized FAD. The largest crystal dimension is 0.4 mm.

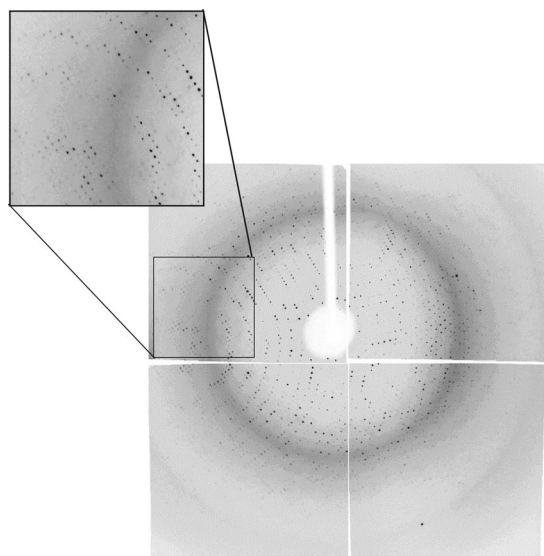


Figure 3
A single 0.25° ω -oscillation frame collected at 100 K on the Brüker CCD detector at APS beamline 17-ID. The enlarged image shows diffraction to 2.2 Å resolution at the detector edge.

was 100.1 mA and decayed to 67 mA over several hours. Data sets were collected at three wavelengths (1.0332, 0.97939, 0.97928 Å) corresponding to the low-energy wavelength, inflection-point wavelength and the peak wavelength as determined by a selenium *K*-edge absorption spectrum. The synchrotron data were collected with the sample under a dry liquid- N_2 -controlled cold stream (Oxford Cryosystems) at 100 K using a Brüker CCD detector at a crystal-to-detector distance of 18 cm and a 2θ of -4.5° . Each of the 500 images of 0.25° ω oscillation was exposed for 2 s. Two-dimensional images were integrated and scaled with the *SAINT* (v.5) software system (Siemens, 1993) in the 20.0–2.3 Å resolution range; unit-cell parameters were $a = b = c = 178.99$ Å in space group $I2_13$ or $I23$ (Fig. 3). The fragile crystals are 81.3% solvent. Data-collection statistics for a typical data set are shown in Table 1. The average mosaic spread of the data was 0.6° . The MAD-phased structure-determination statistics are summarized elsewhere (Benson *et al.*, 2001).

3. Conclusions

MurB protein proved very sensitive to aging and required freshly prepared protein for all crystallization experiments. Sparse-matrix screens and optimization with MurB at 20 mg ml^{-1} revealed one crystallization condition consisting of PEG 8000, $(\text{NH}_4)_2\text{SO}_4$, sodium cacodylate pH 6.5 and 5 mM BME. Single crystals suitable for X-ray diffraction analysis were obtained, but grew firmly attached to the bridges. This problem was prevented by coating the microbridges with a thin layer of vacuum grease prior to the setup of drops. None of the preliminary crystals diffracted to better than 3.5 Å using synchrotron radiation; cocrystallization with a wide variety of additives and substrates did not improve diffraction. Crystals grown with 10 mM EP-UDPGlcNAc substrate resulted in a chunky crystal instead of hexagonal plates and although this substrate analog may have helped in the crystallization of MurB, it was never observed in the crystal structure (Benson *et al.*, 2001). Addition of DMSO (20%) during crystallization improved diffraction to 2.3 Å.

Using these new crystallization conditions, MurB crystals were obtained and optimized with both native and Se-methionine-enriched protein under nearly identical conditions. The Se-methionine crystallization conditions include 0.1 M sodium cacodylate, 9.75% PEG 8000, 0.55 M $(\text{NH}_4)_2\text{SO}_4$, 20% DMSO and 5 mM BME. This mother liquor proved to be an excellent cryopreservative and crystals could be directly looped out of the drop and cryocooled in liquid propane for low-temperature data collection. Data were collected at the Advanced Photon Source (APS) beamline 17-ID. The crystal diffracted to just beyond 2.3 Å resolution, with unit-cell parameters $a = b = c = 178.993$ Å in space group $I2_13$. Data collected from this crystal were used to solve the protein structure (Benson *et al.*, 2001).

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