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FOR THE RECORD

Crystallization and preliminary X-ray crystallographic studies of UDP-*N*-acetylenolpyruvylglucosamine reductase

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Abstract: The overexpression and purification of the second enzyme in *Escherichia coli* peptidoglycan biosynthesis, UDP-*N*-acetylenolpyruvylglucosamine reductase (MurB), provided sufficient protein to undertake crystallization and X-ray crystallographic studies of the enzyme. MurB crystallizes in 14–20% PEG 8000, 100 mM sodium cacodylate, pH 8.0, and 200 mM calcium acetate in the presence of its substrate UDP-*N*-acetylglucosamine enolpyruvate. Crystals of MurB belong to the tetragonal space group $P4_12_12$ with $a = b = 49.6$ Å, $c = 263.2$ Å, and $\alpha = \beta = \gamma = 90°$ at −160 °C and diffract to at least 2.5 Å. Screening for heavy atom derivatives has yielded a single site that is reactive with both methylmercury nitrate and Thimerosal.

Keywords: crystallization; peptidoglycan; UDP-*N*-acetylenolpyruvylglucosamine reductase; X-ray crystallography

The bacterial cell wall serves as a rigid scaffold to maintain the osmotic integrity of the cell. Disruption in the synthesis or breech in the integrity of this biopolymer results in cell lysis and death. Therefore, the multistep pathway catalyzing the synthesis, translocation, and crosslinking of peptidoglycan has been of interest to those concerned with the development of strategies to combat bacterial infection. The first 2 enzymes in the committed biosynthetic pathway in *Escherichia coli* are UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) and UDP-*N*-acetylenolpyruvylglucosamine reductase (MurB). These enzymes catalyze the formation of UDP-*N*-acetylmuramic acid (UDP-MurNAc) in 2 steps by the transfer of an enolpyruvyl moiety from phosphoenolpyruvate and subsequent reduction of the enol ether in the intermediate UDP-*N*-acetylglucosamine enolpyruvate (UDPGlcNAcEP) using 1 equivalent of NADPH (Bugg & Walsh, 1993). The carboxylate of the muramic acid moiety in UDPMurNAc serves as the point of attachment for the first amino acid in the pentapeptide chain, and the *N*-acetylmuramic acid is one of the 2 sugars that comprise the repeating glycan polymer of peptidoglycan. The repeating subunit is formed by transferring the UDPMurNAc pentapeptide to an undecaprenyl lipid carrier with release of UMP and then coupling a molecule of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine. The disaccharide-pentapeptide unit is then translocated across the membrane for the transglycosylation and transpeptidation steps.

The recent cloning of the *murb* gene (Pucci et al., 1992) and the availability of the UDPGlcNAcEP substrate from work on the preceding enzyme MurZ (MurA) (Marquardt et al., 1992) allowed overexpression and purification of 100-mg quantities of MurB for biochemical studies (Benson et al., 1993). MurB purified as a monomer with a molecular weight of 35 kDa from gel filtration analysis. The intense yellow color led to investigation of a possible bound flavin. HPLC analysis of the released chromophore and determination of protein and flavin concentrations showed that MurB contains 1 flavin adenine dinucleotide (FAD) cofactor per molecule of enzyme. Preparations of apoenzyme showed significantly reduced activity, which can be reconstituted with exogenous FAD, corroborating that MurB utilizes the FAD during catalysis (unpubl. results). A probable mechanistic pathway of transfer of the 4-*pro*S hydride from NADPH to the UDPGlcNAcEP substrate via reduced FAD using 4-*S*-^{2}H-NADPH has been defined by NMR analysis of the deuterated UDPMurNAc product. The second proton necessary for reduction comes from a hypothesized solvent exchangeable basic residue determined by analysis of the deuterated UDPMurNAc after catalysis in ^2H_2O (Benson et al., 1993).

An X-ray crystallographic analysis of MurB has been undertaken to ultimately characterize the UDPGlcNAcEP binding site, determine the amino acids involved in catalysis, assess the relative orientation of the substrates, and enhance our understanding of enzymatic reduction of enol ethers. This report describes the crystallization of the enzyme in the presence of its substrate UDPGlcNAcEP and preliminary X-ray studies on native and derivative crystals.

Initial efforts to crystallize MurB were unsuccessful until another column step was introduced into the previously published purification (Benson et al., 1993). MurB bound to a 2′,5′-ADP

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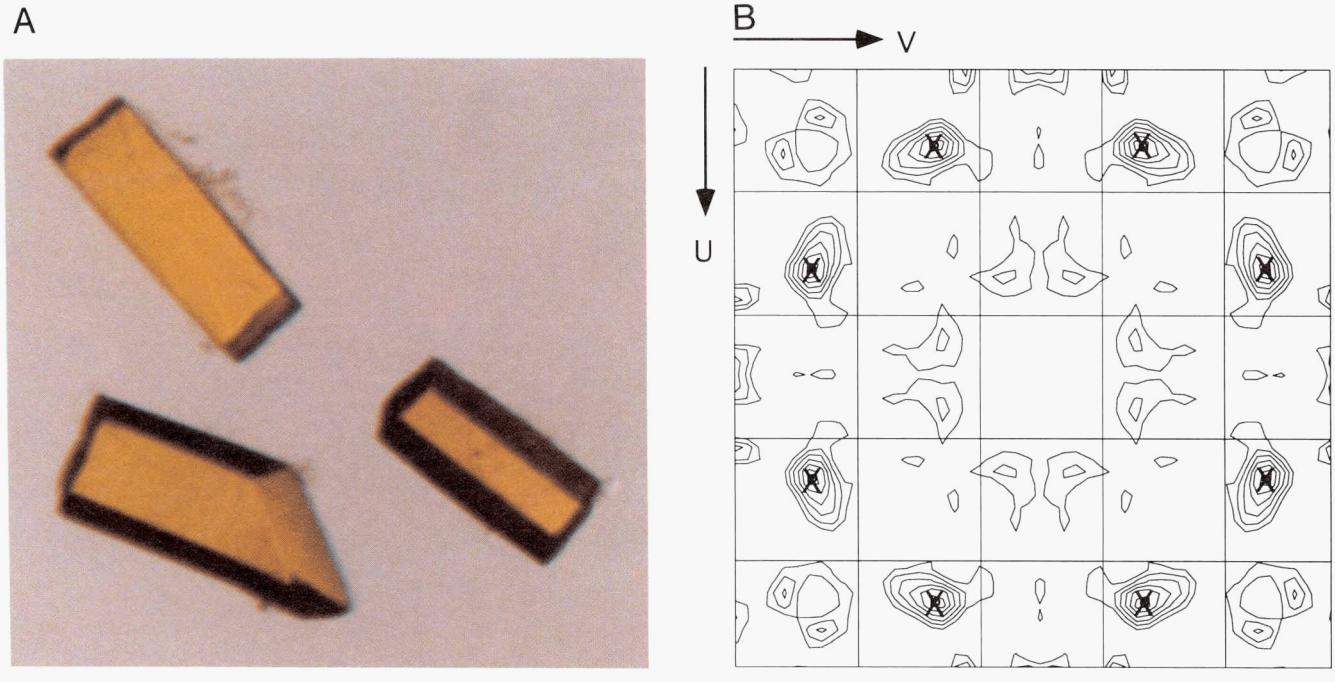

Fig. 1. A: Crystals of MurB in the presence of UDPGlcNAcEP. **B:** Methyl mercury nitrate derivative difference Patterson, 12–3 Å, w section 0.5, contoured at 1σ intervals starting at 1σ above the mean density. This peak is consistent with a heavy atom at position $x = 0.063$, $y = 0.164$, $z = 0.287$. The X's mark the positions of the calculated Patterson peaks based on this site.

Sepharose column (Sigma Chemical Co.) at a pH of 6.4 and eluted in 0.5 M KCl, 50 mM Tris, 0.5 mM DTT, pH 6.4. Several fractions were >95% pure by SDS-PAGE analysis. The first crystals of MurB were obtained in hanging drops using the Hampton Crystal Screen Kit (Riverside, California) (Jancarik & Kim, 1991) at a protein concentration of 20 mg/mL in 18% PEG 8000, 100 mM sodium cacodylate, pH 6.5, 200 mM calcium acetate, and 7 mM UDPGlcNAcEP. Further screening led to optimal conditions of a protein concentration of 10 mg/mL, 14–20% PEG 8000, 100 mM sodium cacodylate, pH 8.0, 200 mM calcium acetate, 7 mM UDPGlcNAcEP. The required PEG 8000 concentration varied depending on the batch of enzyme used. Presence of the substrate UDPGlcNAcEP was absolutely required to obtain crystals. UDPGlcNAcEP was synthesized enzymatically using UDP-*N*-acetylglucosamine, phosphoenolpyruvate, and MurZ (MurA) and purified by HPLC as described (Benson et al., 1993). Crystals also could be grown when HEPES, pH 8.0, and Tris-HCl, pH 8.0, buffers replaced the sodium cacodylate buffer. Crystals formed in a period of 1–2 days, reaching a size of 0.1–0.2 mm in length on each side (Fig. 1A).

Table 1. *Native data collected from a single crystal at −160 °C*

Resolution (Å)	R_{sym}[a]	I/σ	$N_{measurements}$	$N_{reflections}$	% Complete	Multiplicity
∞–7.06	0.035	17.1	3,646	637	97.9	5.7
5.00	0.053	13.2	7,349	1,049	99.4	7.0
4.08	0.053	13.5	8,885	1,299	99.1	6.8
3.53	0.072	10.1	9,080	1,502	99.1	6.0
3.16	0.114	6.7	9,044	1,654	97.7	5.5
2.89	0.182	4.3	7,655	1,595	88.2	4.8
2.67	0.264	3.0	6,365	1,541	79.7	4.1
2.50	0.339	2.3	2,771	1,185	57.9	2.3
Totals	0.077	9.4	54,795	10,462	86.4	5.4

[a] $$R_{sym} = \frac{\sum_h \sum_i |I_{h,i} - \langle I_h \rangle|}{\sum_h \sum_i I_{h,i}},$$

where $\langle I_h \rangle$ is the mean intensity of the i observations for a given reflection h.

Native crystals diffracted to a resolution of at least 2.5 Å (Table 1). Native data were collected both at 25 °C and −160 °C using 2- or 3-min exposures with a frame width of 0.1° on a Siemens rotating anode X-ray generator and multiwire detector. The lifetime of crystals was 2 days during data collection at 25 °C and greater than 7 days at −160 °C in a stream of liquid nitrogen. Before flash freezing at −160 °C, the crystals were soaked in 17% glycerol, 15% PEG 8000, 100 mM sodium cacodylate or HEPES, pH 8.0, and 200 mM calcium acetate for 5 min. Data frames were processed using BUDDHA (Blum et al., 1987) to calculate integrated intensities and the CCP4 program suite (CCP4, 1979) for batch scaling, Patterson calculations, and plotting. The unit cell dimensions are $a = b = 50.3$ Å, $c = 270.3$ Å at 25 °C and $a = b = 49.6$ Å, $c = 263.2$ Å at −160 °C with $\alpha = \beta = \gamma = 90°$. Diffraction data collected from these crystals exhibit equivalences characteristic of the 4/mmm Laue group. The space group was determined to be $P4_12_12$ (or its enantiomorph $P4_32_12$) based on systematic absences of the OO*l* reflections (only reflections with $l = 4n$ are present) and of the *h*OO and O*k*O reflections (only reflections with h or $k = 2n$ are present). This space group assignment has been confirmed by the unique solution of the Patterson for the first 2 derivatives in $P4_12_12$. Given a calculated molecular weight of 37,854 Da and 8 asymmetric units in the unit cell, the Matthews coefficient is 2.14, which is consistent with the presence of a single molecule in the asymmetric unit and a solvent content of 48%.

Initial heavy atom screening was conducted in soaking experiments with heavy metal solutions (1 mM) in sodium cacodylate crystallization buffer. Because of the reduced buffering capacity of cacodylate buffer at pH 8.0, HEPES buffer is currently used in the search for derivatives. Several mercurials cracked MurB crystals within a few hours, but co-crystallization of MurB with methylmercury nitrate or Thimerosal gave substitution at a single site. The methylmercury nitrate derivative was isomorphous to 3 Å (Fig. 1B). Other compounds are being investigated to find other derivatives.

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