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Crystallization and preliminary crystallographic analysis of *E. coli* uridine 5′-diphospho-*N*-acetylenolpyruvylglucosamine reductase in two new crystal forms

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Abstract

Uridine 5′-diphospho-*N*-acetylenolpyruvylglucosamine reductase (MurB), the second enzyme in the peptidoglycan synthetic pathway of *Escherichia coli*, has been crystallized in two previously unreported forms, one orthorhombic and the other monoclinic. MurB (molecular mass 38 kDa) crystallizes in a range of conditions that utilize polyethylene glycol fractions as precipitants, and crystals can be grown with or without the enzyme's substrate, uridine 5′-diphospho-*N*-acetylenolpyruvylglucosamine. X-ray diffraction from crystals of the orthorhombic form extends to 2 Å resolution and shows the symmetry and systematic absences of space group $P2_12_12_1$. These crystals show significant variations in cell dimensions at room temperature and at 100 K. A crystal used to collect a 2.0 Å resolution data set at a synchrotron source showed cell dimensions at *ca* 100 K of $a = 51.0$, $b = 79.3$ and $c = 87.1$ Å, indicating one molecule per asymmetric unit. The monoclinic crystals scatter X-rays to 3.0 Å resolution consistent with space group $P2_1$, unit-cell dimensions (*ca* 100 K) $a = 50.7$, $b = 92.4$, $c = 85.5$ Å, and $\beta = 104°$, and two molecules per asymmetric unit. Mercury derivatives have been prepared with both orthorhombic and monoclinic forms, and efforts are underway to exploit these derivatives to determine the structure of this protein.

1. Abbreviations

CCD, charge-couple device; CHESS, Cornell High Energy Synchrotron Source; EMBL, European Molecular Biology Laboratory; FAD, flavin adenine dinucleotide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonate); MAD, multi-wavelength anomalous dispersion; MBP, maltose-binding protein; *m*-DAP, *meso*-diaminopimelate; NADPH, β-nicotinamide adenine dinucleotide 3′-phosphate; NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; NSLS, National Synchrotron Light Source; PCMB, *p*-chloromercuribenzoate; PEG, polyethylene glycol; UDP-, uridine 5′-diphospho-; UNAG, UDP-*N*-acetylglucosamine; UNAGEP, UDP-*N*-acetylenolpyruvylglucosamine; UNAM, UDP-*N*-acetylmuramic acid.

2. Introduction

The search for new antibiotics is an important area of research, where new strategies are sought to treat drug-resistant strains of bacterial infections that are appearing with increasing frequency. The enzymes responsible for synthesis of the peptidoglycan layer of the bacterial cell wall, unique to prokaryotes, make attractive, highly specific, and in some cases proven antibiotic drug targets. Uridine 5′-diphospho-*N*-acetylenolpyruvylglucosamine reductase (E.C. 1.1.1.158), the product of the *murb* gene of *Escherichia coli* and hereinafter referred to as MurB, is one in a linked sequence of several enzymes in the biosynthetic pathway that produces this layer.

The peptidoglycan layer, which provides structural strength to the cell wall of Gram-positive and Gram-negative bacteria, is a complex molecular network made primarily of polysaccharide strands. The strands consist of alternating sugar residues, *N*-acetylmuramic acid (NAM) pentapeptide and *N*-acetylglucosamine (NAG), that are cross-linked through the pentapeptide side chains of NAM pentapeptide units (Bugg & Walsh, 1992). MurB is a flavoprotein of about 40 kDa, that catalyzes the second step in the building of this network, the reduction of the enolpyruvate of UDP-*N*-acetylenolpyruvylglucosamine (UNAGEP), with reducing equivalents from NADPH and with enzyme-bound flavin adenine dinucleuotide (FAD) as redox intermediate (Benson, Marquardt, Marquardt, Etzkorn & Walsh, 1993). The product is UDP-*N*-acetylmuramic acid (UNAM), which is then modified by a linked sequence of other *mur*-gene enzymes to build the pentapeptide precursor UNAM-L-Ala-γ-D-Glu-*m*-DAP-D-Ala-D-Ala.

3. Preparation and purification

MurB was prepared and purified by minor modifications of a previously published procedure (Dhalla *et al.*, 1995). Briefly, the enzyme was overexpressed in *E. coli* as a fusion protein C-terminal to maltose-binding protein (MBP) (Pucci, Discotto & Dougherty, 1992). Cells were lysed, lysate was clarified by centrifugation, and fusion product was extracted from supernatant by amylose-resin affinity chromatography. MBP was cleaved from MurB by proteolysis with factor Xa enzyme leaving MurB with an N-terminal extension of four amino acids, and cleavage products were separated by anion-exchange chromatography. Purified enzyme contained tightly bound FAD cofactor and was fully active as purified. Expected mass based on cDNA including N-terminal extension was verified by electrospray mass spectrometry.

Three different variants of the MurB enzyme, each with the four-residue N-terminal extension, were prepared as above: a wild-type MurB (38336 Da); an accidental mutant, Q130H, in which the glutamine at residue 134 of wild-type MurB is replaced by histidine; and a selenomethionylated MurB, SeMet-MurB, in which all five methionines of wild-type MurB are replaced by selenomethionyl residues by expression in an *E. coli* auxotroph. Electrospray mass spectrometry of samples of SeMet-MurB showed 100% substitution of selenomethionine at each of the five positions. All three variants are fully active in enzyme assays.

MurB substrate, UDP-*N*-acetylenolpyruvylglucosamine (UNAGEP), was prepared by enzymatic synthesis as previously described (Dhalla *et al.*, 1995). In this method, the first enzyme in the series, UDP-*N*-acetylenolpyruvylglucosamine transferase or MurA, in its fusion protein form, is used to convert UNAG to UNAGEP in the presence of phosphoenolpyruvate.

4. Crystallization and derivative preparation

Pure protein in storage buffer (*bis*-Tris propane buffer, pH 8.0, with 20% glycerol) was concentrated in a centrifuge with Centricon concentrators (Amicon, Beverly, MA, USA) to 15–20 mg ml^{-1} with simultaneous exchange of buffer to 50 m*M* HEPES, pH 8.0, for use in crystallization. Under ambient conditions, the enzyme is in the oxidized state and can bind, but cannot reduce substrate. Protein was used as is, or combined with substrate UNAGEP for crystallization experiments. Mercury co-crystallization was done in two ways: crystallization in the presence of mercury reagents and compounds, and crystallization of MurB repurified after reaction with the mercury reagent *p*-chloromercuribenzoate (PCMB).

Crystals were grown at 293 K by the hanging-drop vapor-diffusion method, in which droplets were composed of protein and reservoir solutions in equal volumes. Crystals were obtained in three different forms, each with more than one variant of the protein in a manner suggesting that, while not specifically verified, each variant of MurB protein described can produce all three of these crystal forms.

The first form we produced was the orthorhombic form, which was obtained from MurB protein in the absence of substrate (although in later experiments orthorhombic crystals were also grown from mixtures of protein and substrate). The best crystals of this form are grown from 15–20%(*w*/*v*) PEG 5000 monomethyl ether (Fluka Chemical Corp., Ronkonkoma NY) in 50 m*M* HEPES buffer, pH 7.25, with 50 m*M* calcium chloride. These crystals grew rapidly in the form of elongated flat plates, reaching diffraction size within a few days, and terminating growth at up to 1.5 × 0.35 × 0.08 mm. Crystals tended to grow in layered clusters in which individuals were thin in the third dimension. Seeding was not found to be useful in obtaining thicker crystals, but the addition of glycerol (or a combination of glycerol and ethanol) to the growth medium often proved helpful. Interestingly, crystal growth habit was also affected by droplet shape, drop volume, and protein concentrations; it was observed that crystals initiating growth at the edges of the droplets often grew thicker, so that artificial creation of elongated extensions off the main droplet, or uneven droplet edges, often resulted in growth of thicker crystals. All three variants of MurB protein were used to produce orthorhombic crystals; wild type and Q130H were indistinguishable in these experiments and, if anything, SeMet-MurB gave better crystals than the others.

Our initial crystallographic efforts concentrated on the orthorhombic form, which we used in a search for heavy-atom derivatives, and it was in this search that we discovered other crystal forms. The monoclinic crystal form was discovered in attempts to crystallize MurB with UNAGEP substrate in the presence of mercury reagents. In these experiments, a 15–20× molar excess of UNAGEP was incubated with 20 mg ml^{-1} protein for a few hours at room temperature, then stored at 277 K for later use. For mercury co-crystallization, mercury acetate was added to the droplet at a final concentration of approximately 1 m*M*. The best crystals of the monoclinic form were grown in a mixture of 8.0–9.5% PEG 8000 (Hampton Research, Riverside, CA, USA) and 0.2 *M* calcium acetate in 0.1 *M* HEPES buffer, pH 8.0. Monoclinic crystals also grow as thin plates over a period of weeks, but macroseeding was required to produce monoclinic crystals of useful size, the maximum being about 0.4 × 0.4 × 0.02 mm. For a number of reasons, it became important to grow these crystals without mercury reagent. We were successful in this after many tries at conditions similar to those above, but in general crystals were of poor quality and small and the harvest of useful specimens was meager.

The monoclinic crystal form often appeared with another form in the same droplet, although this was not true of all batches of protein. This other form could be grown under the same crystallization conditions as the monoclinic form, and a crystal size of about 0.3 × 0.2 × 0.2 mm could be attained with macroseeding. This crystal form proved to be identical to the tetragonal form reported by Benson and colleagues (Benson, Walsh & Hogle, 1994) and we later discovered the tetragonal form in some droplets containing UNAGEP that produced orthorhombic crystals.

In the effort to produce a mercury derivative with the orthorhombic form, MurB was reacted with PCMB (Pfaltz and Bauer, Waterbury, CT, USA), run over a PD10 desalting column (Pharmacia, Piscataway, NJ, USA), and then used in crystallization. This material readily and reproducibly provided crystals of the orthorhombic form under conditions similar to those used with the native form. Analysis by electrospray mass spectrometry showed addition of from one to five PCMB molecules per molecule of MurB, with no unsubstituted MurB protein.

5. Data collection and analysis

Data collection was carried out at room temperature on crystals mounted in thin-wall glass capillaries, and on flash-cooled crystals at *ca* 100 K. Crystals were prepared for flash-cooling by transferring them through stabilization precipitant solutions containing increasing concentrations of glycerol up to 25%. Crystals were picked up in a fiber loop and flash-cooled in a dry nitrogen cold stream or by direct immersion in liquid nitrogen (Rodgers, 1994).

Crystal data were collected on a Siemens X-1000 area detector mounted on a Rigaku copper rotating-anode source equipped with focussing mirror optics, and rocking curves of individual reflections were examined with Cu $K\alpha$ radiation to determine stabilization conditions for flash-cooling. Unit-cell dimensions for the orthorhombic form were found to vary significantly from crystal to crystal. Estimations of unit-cell dimensions for about 40 crystals, half at *ca* 295 K and half at *ca* 100 K, show that distributions about means at the two temperatures are almost non-overlapping (Table 1). Such variation in fundamental dimensions among crystals at a given temperature poses significant problems in avoiding non-isomorphism. The monoclinic crystals did not show the wide variation in estimates of unit-cell dimensions found for the orthorhombic form. Diffraction characteristics of examples we grew of the tetragonal crystal form accord well with those reported (Benson *et al.*, 1994).

We have not been able to find a heavy-atom derivative of the orthorhombic form by standard soaking procedures. Accord-

Table 1. *MurB crystal forms*

Form	Space group	Temperature (K)	a (Å)	b (Å)	c (Å)	β (°)	No./ a.u.*	V_M† (Å^3 Da^{-1})	Resolution (Å)
1	$P2_12_12_1$	*ca* 295	50.8 (0.5)	80.5 (1.2)	87.1 (1.1)	—	1	2.3	2.6
	$P2_12_12_1$	*ca* 100	50.1 (0.9)	78.6 (1.1)	85.0 (1.1)	—	1	2.2	2.0
2	$P2_1$	*ca* 100	50.7	92.4	85.5	104	2	2.5	3.0
3	$P4_12_12$‡	*ca* 100	49.7	49.7	263.5	—	1	2.1	2.6

* Number of molecules per asymmetric unit. † Crystal volume per Da (Matthews, 1968). ‡ Or enantiomer.

ingly, we have explored possible routes to structure solution by multiwavelength anomalous dispersion (MAD) techniques. The synthesis of the SeMet-MurB protein, in which all five methionines in the enzyme are replaced with selenomethionines, was one part of this effort. The first orthorhombic crystal of SeMet-MurB, mounted in a thin-wall glass capillary and examined on an R-AXIS IIc with Yale (Molecular Structure Corporation) mirror optics, diffracted to at least as high a resolution at room temperature as native (SMet) MurB. A three-wavelength MAD data set (inflection and peak of the fluorescence spectrum, and remote) was collected at CHESS beamline F2 at Cornell University with a CCD detector (Tate *et al.*, 1995) on a flash-cooled SeMet-MurB crystal. Data were reduced with the *HKL* package (Otwinowski, 1993; Minor, 1993). Maps and phases were calculated with the program *PHASES* (Furey & Swaminathan, 1990). Examination of Patterson maps and Bijvoet difference Fourier maps showed the location of four Se sites (the absence of a fifth Se site is presumably due to lack of order of the selenomethionyl residue at the N-terminus).

A second three-wavelength MAD data set (inflection, peak and remote wavelengths) was collected at NSLS beamline X4A with Fuji image plates on a flash-cooled crystal of PCMB-treated MurB. These data were also reduced with the *HKL* package. Examination of anomalous and dispersive Patterson maps showed the presence of two Hg atoms at approximately equal occupancy. Although the cell edges of the crystal used for this data set differed from those of the crystal used for SeMet MAD phasing, cross-dispersive and Bijvoet (anomalous) difference Fouriers showed mercury sites that were in agreement with the Patterson maps. Structure analysis of the orthorhombic form continues.

Data have also been collected from room-temperature and flash-cooled crystals of the monoclinic form grown both in the presence and absence of methyl mercury acetate. A Patterson synthesis based solely on data from a crystal grown with mercury shows a strong peak (60% of the origin peak) at $(0,0,\frac{1}{2})$, suggesting that the two molecules in the asymmetric unit are related by a translation of $\frac{1}{2}$ along c. A difference Patterson calculated from data from crystals grown with and without mercury corroborates this suggestion and the major peaks in this map can be explained by a pair of Hg-atom positions related by the translation of $\frac{1}{2}$ in c. The similarity in a and c dimensions of the orthorhombic and monoclinic forms suggests that the packing schemes in these two forms may be similar, and may be further related to that of the tetragonal form given its a dimension (Table 1). We continue to pursue opportunities for structure solution based on the monoclinic form.

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References

Benson, T. E., Marquardt, J. L., Marquardt, A. C., Etzkorn, F. A. & Walsh, C. T. (1993). *Biochemistry*, **32**, 2024–2030.

Benson, T. E., Walsh, C. T. & Hogle, J. M. (1994). *Protein Sci.* **3**, 1125–1127.

Bugg, T. D. H. & Walsh, C. T. (1992). *Nat. Prod. Rep.* **9**, 199–215.

Dhalla, A. M., Yanchunas, J. Jr, Ho, H.-T., Falk, P. J., Villafranca, J. J. & Robertson, J. G. (1995). *Biochemistry*, **34**, 5390–5402.

Furey, W. & Swaminathan, S. (1990). Am. Crystallogr. Assoc. Meet. Ser. 2, Vol. 18, p. 73, Abstract PA33.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Minor, W. (1993). *XdisplayF Program.* Purdue University, West Lafayette, IN, USA.

Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend: Data Collection and Processing*, pp. 56–62. Warrington: Daresbury Laboratory.

Pucci, M. J., Discotto, L. F. & Dougherty, T. J. (1992). *J. Bacteriol.* **174**, 1690–1693.

Rodgers, D. W. (1994). *Structure*, **2**, 1135–1140.

Tate, M. W., Eikenberry, E. F. Barna, S. L., Wall, M. E., Lowrance, J. L. & Gruner, S. M. (1995). *J. Appl. Cryst.* **28**, 196–205.