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CRYSTALLIZATION NOTE

Crystallization and Preliminary X-Ray Diffraction Analysis of UDP-*N*-acetylglucosamine Enolpyruvyltransferase of *Enterobacter cloacae*

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Single crystals of UDP-*N*-acetylglucosamine enolpyruvyltransferase of *Enterobacter cloacae* have been grown by vapor diffusion using phosphate buffer as the precipitant. The crystals belong to the monoclinic space group *C*2 with *a* = 86.9 Å, *b* = 155.9 Å, *c* = 83.8 Å, β = 91.6°. Assuming two monomers per asymmetric unit, the solvent content of these crystals is 63%. Flash-frozen crystals diffract to beyond 2 Å resolution. © 1996 Academic Press, Inc.

INTRODUCTION

The bacterial cell wall imparts rigidity to bacteria and stabilizes them to their high internal osmotic pressure. The major structural element of the wall is the peptidoglycan murein. This macromolecule is a heteropolymer consisting of alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid, crosslinked by peptide chains which are attached to the lactate group of the muramic acid residues. The branch-point between general nucleotide-sugar metabolism and *N*-acetylmuramyl peptide synthesis is the enolpyruvyl transfer from phosphoenolpyruvate (PEP) to the 3-hydroxyl of UDP-*N*-acetylglucosamine (UDP-GlcNAc), catalyzed by UDP-*N*-acetylglucosamine enolpyruvyltransferase (EPT, EC 2.5.1.7) (Scheme 1). EPT is of potential pharmaceutical interest because it is irreversibly inhibited by the natural antibiotic fosfomycin [(1*R*,2*S*)-1,2-epoxypropyl phosphonic acid] (Kahan *et al.,* 1974). For instance, fosfomycin trometamol salt is an orally administered antibiotic that may be used for single-dose therapy of uncomplicated urinary tract infections (Scaglione *et al.,* 1994). Recently, the mechanism of action of fosfomycin on EPT has been reevaluated using the large quantities of enzyme available from overexpression of *Escherichia coli* EPT (Marquardt *et al.,* 1994) and *Enterobacter cloacae* EPT (Wanke and Amrhein, 1993).

The only other enzyme known to catalyze the transfer of the intact enolpyruvyl moiety of PEP to a substrate is the EPSP synthase (3-phosphoshikimate 1-carboxyvinyltransferase, EC 2.5.1.19), the sixth enzyme of the shikimate pathway and the target of glyphosate, the active ingredient of the broad-spectrum herbicide Roundup (Steinrücken and Amrhein, 1980).

Even though the two enzymes share significant similarities in their amino acid sequences (25%

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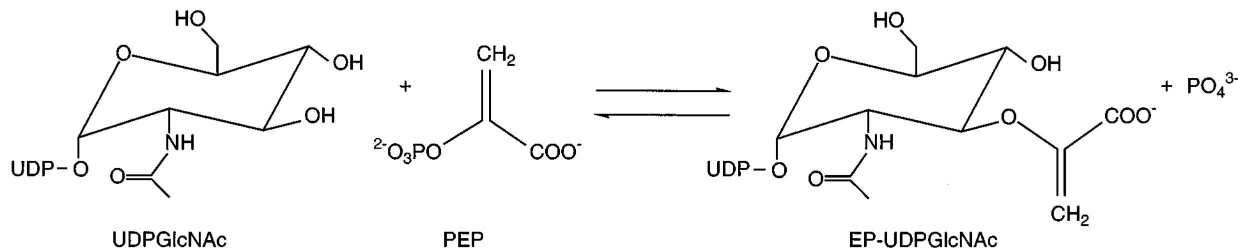

SCHEME 1. The reaction catalyzed by EPT. The enolpyruvyl moiety of phosphoenolpyruvate (PEP) is transferred to UDP-*N*-acetylglucosamine (UDPGlcNAc) to form enolpyruvyl-UDP-*N*-acetylglucosamine (EP-UDPGlcNAc).

identity and 47% similarity; Wanke *et al.,* 1992), their reaction mechanisms appear to be substantially different. The reaction pathway of EPT appears to involve the formation of a covalent intermediate, in which PEP is attached to the enzyme as the *O*-phosphothioketal of pyruvate through cysteine-115 (Wanke and Amrhein, 1993; Ramilo *et al.,* 1994). In contrast, the EPSP synthase reaction proceeds through a tightly, but noncovalently, bound tetrahedral intermediate (Anderson and Johnson, 1990). Recent studies of Brown *et al.* (1994) on the *E. coli* EPT have provided some evidence that the formation of the enzyme-bound *O*-phosphothioketal is followed by the formation of a tetrahedral intermediate. Interestingly, EPSP synthase is not inactivated by fosfomycin and glyphosate exerts no effect on EPT (Steinrücken and Amrhein, 1984).

The crystal structure of the native EPSP synthase is known at 3 Å resolution (Stallings *et al.,* 1991). It is a two-domain structure with a unique fold that appears to be formed by a sixfold replication of a protein folding unit comprising two parallel helices and a four-stranded sheet. The authors proposed that this may be the result of a gene duplication of a primordial 70-amino-acid unit. Marquardt *et al.* (1992) searched for a repeating unit in the amino acid sequence of *E. coli* EPT and found a conserved motif which is present in six units of approximately 70 amino acids, suggesting that the two enzymes might be built according to a similar plan.

Clearly, the knowledge of the 3D structure of EPT will contribute to a better understanding of structural and catalytic homologies between EPT and EPSP synthase. Furthermore, a detailed structural analysis of EPT may facilitate the development of novel antibacterial agents.

We report here the crystallization and preliminary X-ray analysis of EPT in the absence of substrate and inhibitor.

METHODS AND RESULTS

Cloning, overexpression, and purification of *E. cloacae* EPT have been described by Wanke *et al.* (1992).

The enzyme was transferred to 50 m*M* sodium/potassium phosphate buffer (pH 6.9) containing 1 m*M* DTT using a PD-10 column (Pharmacia) and concentrated to 60 mg/ml via Centricon-30 (Amicon).

Crystals were grown by the hanging drop vapor diffusion method (McPherson, 1990) using plastic tissue culture plates. Droplets typically composed of 5 μl of enzyme and 5 μl of 0.8 *M* sodium/potassium phosphate buffer (pH 6.4) containing 40 m*M* cyclohexylammonium phosphate were equilibrated against 1 ml 0.8 *M* sodium/potassium phosphate buffer (pH 6.4). Crystallization usually occurred within 3 days and the crystals reached their maximum size of $0.5 \cdot 0.5 \cdot 0.1$ mm^3 after 5 days at room temperature (Fig. 1). In the absence of cyclohexylammonium ions, crystals grew as thin needles which were not useful for crystallographic analysis.

Initial X-ray diffraction experiments were performed at room temperature with crystals mounted in glass capillaries at the EMBL X31 beamline (DESY, Hamburg). Under these conditions, the maximal resolution observed was 2.5 Å. The reflections could be indexed on a monoclinic lattice (*C*2) with unit cell parameters $a = 89.6$ Å, $b = 156.8$ Å, $c = 84.6$ Å, and $\beta = 91.2°$.

Since the crystals are radiation sensitive we established conditions suitable for flash freezing (Teng, 1990). The cryoprotectant consisted of 1.5 *M* sodium/potassium phosphate buffer (pH 6.4) containing 40 m*M* cyclohexylammonium phosphate and 35% glycerol. Crystals were soaked in the cryoprotectant for less than 1 min and were then mounted in loops made from dental floss. Frozen crystals showed a significant increase in resolution with only a slight increase in mosaic spread. Using the same exposure time for frozen crystals and for crystals kept at room temperature the resolution increased from the earlier 2.5 Å to 1.8 Å. Moreover, prolonged exposure times at the EMBL X11 beamline resulted in a further increase in resolution up to 1.3 Å. The unit cell parameters at 120 K are $a = 86.9$ Å, $b = 155.9$ Å, $c = 83.8$ Å, $\beta = 91.6°$.

Data were collected from a single crystal at the EMBL X31 beamline (DESY, Hamburg) with a MAR

FIG. 1. Crystals of EPT. See text for detailed crystallization conditions (bar, 0.15 mm).

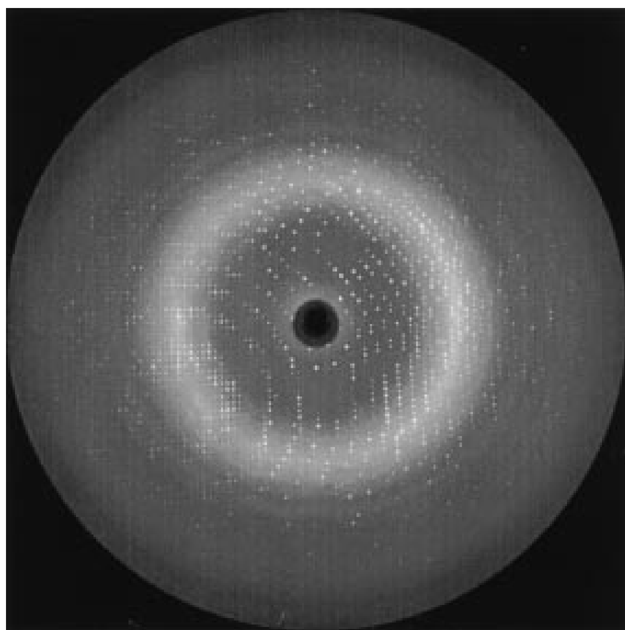

FIG. 2. X-ray diffraction pattern from an EPT crystal recorded on the X31 EMBL beamline (DESY, Hamburg). The oscillation range was 1°, wavelength 0.99 Å, exposure dose 7500 counts, crystal-to-detector distance 140 mm. The edge of the image corresponds to 1.8 Å resolution.

image plate detector using a wavelength of 0.99 Å (Fig. 2). The oscillation ranges were 1° and 3° for high- and low-resolution data, respectively. The raw data were processed with the DENZO-SCALEPACK package (Otwinowski, 1993).

The number of total observations between 25 and 1.8 Å was 744, 941. These were reduced to 103, 189 independent reflections. The data set was complete to 99.8% in this resolution range, the overall R_{sym} was 5.3% rising to 29% in the highest resolution shell.

The V_m value (Matthews, 1968) was calculated to be 3.3 Å^3/Da assuming that two monomers EPT (M_r = 44, 776) are present in the asymmetric unit. This corresponds to a solvent content of 63%. Self-rotation function using the program POLARRFFN (CCP4, 1994) revealed a peak at $\kappa = 180°$, $\omega = 85.2°$, $\phi = 0°$ that can be interpreted as a twofold noncrystallographic symmetry. This supports the estimate of two monomers per asymmetric unit from the calculation of V_m. The structure determination using these crystals is currently underway.

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