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Crystallization and preliminary X-ray crystallographic studies of 3-deoxy-*manno*-octulosonate cytidylyltransferase from *Haemophilus influenzae*

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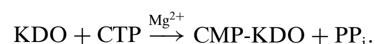
The enzyme 3-deoxy-*manno*-octulosonate cytidylyltransferase (CMP-KDO synthetase; CKS) catalyzes the activation of 3-deoxy-*manno*-octulosonate (KDO) by forming CMP-KDO. It is essential for the biosynthesis of lipopolysaccharides in Gram-negative bacteria and is a potential target for the discovery of antibacterial agents. L-CKS from *Haemophilus influenzae* was overexpressed with a C-terminal hexahistidine tag in *Escherichia coli* and crystallized in the presence of the substrate KDO at 297 K using PEG 4000 as a precipitant and ethylene glycol as an additive. The diffraction limit and spot shape of the native crystal could be improved significantly by dehydration/annealing. X-ray diffraction data were collected to 2.5 Å resolution from a native crystal. The crystals are orthorhombic, belonging to the space group $P2_12_12_1$, with unit-cell parameters a = 48.6, b = 83.1, c = 117.3 Å. The presence of two monomers of recombinant L-CKS in the crystallographic asymmetric unit gives a reasonable V_M of 2.05 Å^3 Da^{-1}, with a solvent content of 40.0%.

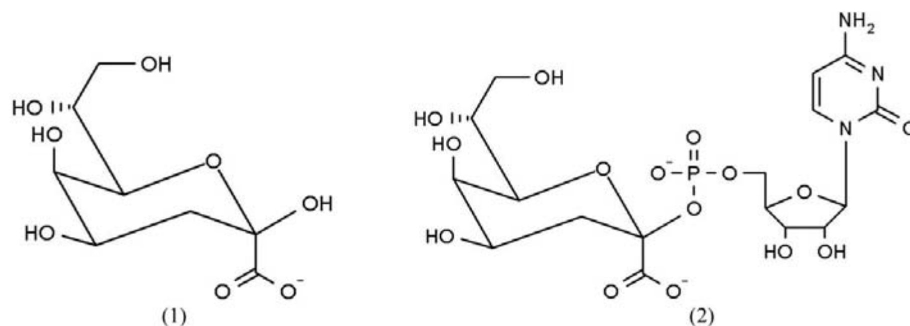
1. Introduction

The sugar 3-deoxy-D-*manno*-octulosonate [or 2-keto-3-deoxy-*manno*-octonic acid, KDO (1)] is an essential component of lipopolysaccharides (LPS) in Gram-negative bacteria. It serves as the link between lipid A and the core oligosaccharides of LPS (Raetz, 1996). Incorporation of KDO into lipopolysaccharides is catalyzed by the membrane-bound KDO transferase(s) that require(s) a nucleotide derivative of the sugar, CMP-KDO (2) (Royo *et al.*, 2000). Synthesis of CMP-KDO is catalyzed by 3-deoxy-*manno*-octulosonate cytidylyltransferase (CMP-KDO synthetase; CKS; E.C. 2.7.7.38) according to the reaction



$$\mathrm{KDO} + \mathrm{CTP} \xrightarrow{\mathrm{Mg}^{2+}} \mathrm{CMP\text{-}KDO} + \mathrm{PP_i}.$$

This reaction is unique to Gram-negative bacteria and therefore absent from mammalian cells (Jelakovic & Schulz, 2001). Therefore, CKS is an attractive target for the development of antibacterial agents (Goldman *et al.*, 1987). Two functionally distinct isozymes, L-CKS and K-CKS, have been found in *Escherichia coli*. L-CKS is encoded by the *kdsB* gene and is involved in the biosynthesis of LPS, while K-CKS is encoded by the *kpsU* gene and participates in the capsule expression (Rosenow *et al.*, 1995). These two isozymes show an amino-acid sequence identity of 44% and catalyze the same reaction, but with different kinetic properties and under different conditions in the bacterial cell (Rosenow *et al.*, 1995). In order to elucidate the structural basis of their functional differences, it is necessary to compare their three-dimensional structures. However, structural information is presently available for only K-CKS from *E. coli* (Jelakovic *et al.*, 1996; Jelakovic & Schulz, 2001, 2002). Therefore, we have initiated the structure determination of L-CKS from *H. influenzae* (Fleischmann *et al.*, 1995). The protein is composed of 254 residues with



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headxl;M_r = 28 124 (HI0058; http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl) and shows a high level (63%) of amino-acid sequence identity to L-CKS from *E. coli* (Goldman *et al.*, 1986). In comparison, it shows a lower level (43%) of sequence identity to K-CKS from *E. coli*. In this study, we overexpressed L-CKS from *H. influenzae* with a C-terminal hexahistidine tag in *E. coli* and crystallized it in the presence of the substrate KDO. Here, we report its crystallization conditions and preliminary X-ray crystallographic data.

2. Experimental

2.1. Protein expression and purification

The *kdsB* gene encoding L-CKS was amplified by the polymerase chain reaction using the *H. influenzae* genomic DNA as a template. The sequences of the forward and reverse oligonucleotide primers designed from the published genome sequence (Fleischmann *et al.*, 1995) were 5′-G GAA TTC **CAT ATG** TCA TTT ACC GTG ATT ATC CCC-3′ and 5′-CCG CCG **CTC GAG** ATT CGC CGC TAA AAT TGC CCG C-3′, respectively. The bases in bold represent the *Nde*I and *Xho*I digestion sites, respectively. The amplified DNA was inserted into the *Nde*I/*Xho*I-digested expression vector pET-21a (Novagen). This vector construction has six histidine residues to the C-terminus of the *kdsB* gene product to facilitate protein purification (see below). The protein was overexpressed in *E. coli* B834(DE3) cells. Cells were grown at 310 K to an OD_{600} of 0.5 in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin and protein expression was induced by 0.5 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG). Cell growth continued at 303 K for 4 h after IPTG induction and cells were harvested by centrifugation at 4200*g* (6000 rev min^{-1}; Sorvall GSA rotor) for 10 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer (20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl, 5 m*M* imidazole and 1 m*M* phenylmethylsulfonyl fluoride) and was homogenized with an ultrasonic processor. The crude cell extract was centrifuged at 36 000*g* (18 000 rev min^{-1}; Hanil Supra 21K rotor) for 30 min at 277 K and the recombinant protein in the supernatant fraction was purified by two chromatographic steps. The first step utilized the C-terminal hexahistidine tag by metal-chelate chromatography on Ni-NTA resin (Qiagen). Gel filtration was then performed on a HiLoad XK 16 Superdex 200 prep-grade column (Amersham-Pharmacia), which was previously equilibrated with buffer *A* (50 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA) containing 100 m*M* sodium chloride. Homogeneity of the purified protein was assessed by SDS–PAGE (Laemmli, 1970). The protein solution was concentrated using a YM10 ultrafiltration membrane (Amicon). The protein concentration was estimated by measuring the absorbance at 280 nm, employing the calculated extinction coefficient of 15 930 M^{-1} cm^{-1} (SWISS-PROT; http://www.expasy.ch/).

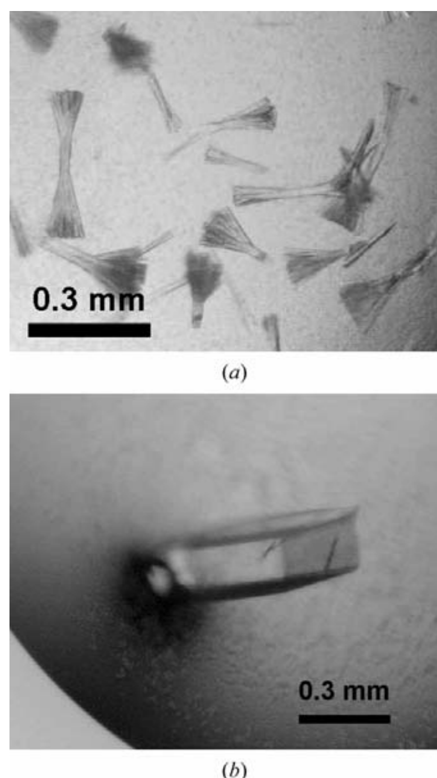

Figure 1
Crystals of CMP-KDO synthetase from *H. influenzae* grown in the presence of its substrate KDO. (*a*) Broom-shaped crystals grown in the absence of ethylene glycol. (*b*) A rectangular crystal grown in the presence of 3.0%(*v*/*v*) ethylene glycol as an additive. Its approximate dimensions are 0.4 × 0.3 × 0.15 mm.

2.2. Crystallization

Crystallization was performed at 297 K by the hanging-drop vapour-diffusion method using 24-well tissue-culture plates. Before the crystallization setup, the substrate KDO was added to the protein solution in 15-fold excess over the subunit concentration and the protein solution was incubated at 277 K for 1 d. Each hanging drop was prepared on a siliconized cover slip by mixing equal volumes (2 µl each) of the protein solution (at 15 mg ml^{-1} protein concentration) and the reservoir solution. When a number of additives were examined to improve the crystal quality, the hanging drop was prepared by mixing 2 µl of the protein solution, 2 µl of the reservoir solution and 0.4 µl of the additive solution, but the additive was not added to the reservoir solution. Each hanging drop was placed over a 1.0 ml reservoir solution. Initial crystallization conditions were established using screening kits from Hampton Research (Crystal Screens I, II and MembFac) and from Emerald BioStructures Inc. (Wizard I and II).

2.3. X-ray diffraction experiment

A crystal of the native protein was flash-frozen with a cryoprotectant solution, which consisted of 100 m*M* sodium citrate pH 5.6, 50 m*M* ammonium acetate, 30%(*w*/*v*) PEG 4000 and 20%(*v*/*v*) PEG 400. Crystals were soaked in 5 µl of the cryoprotectant solution for 10 s before being flash-frozen in liquid nitrogen. Since the crystal gave elongated spot shapes upon exposure to X-rays, it was removed from the cold nitrogen-gas stream and placed in 100 µl of the cryoprotectant solution in a spot plate (Hampton Research) without sealing and the cryoprotectant solution was dried out slowly for about 30 min. The dehydrated and annealed crystal was again flash-cooled. X-ray diffraction data were collected at 100 K with an ADSC Quantum 4R CCD detector at the BL-18B experimental station of the Photon Factory, Japan. The crystal was oscillated by 1.0° per frame over a total range of 108°. Intensity data were processed and scaled using the programs *MOSFLM* (Leslie, 1994) and *SCALA* (Collaborative Computational Project, Number 4, 1994). X-ray data from a crystal of selenomethionine-substituted protein were similarly collected on a MacScience imaging-plate detector at the BL-6B experimental station of Pohang Light Source, Korea, except that the crystal dehydration/annealing step was omitted.

3. Results

The recombinant CKS from *H. influenzae* with a C-terminal hexahistidine tag was highly overexpressed in soluble form in *E. coli* cells, with a yield of ~75 mg of the purified enzyme per litre of culture. Initially, broom-shaped crystals (Fig. 1) were obtained using a reservoir solution at pH 5.6 containing 100 m*M* sodium citrate, 30%(*w*/*v*) PEG 4000 and 50 m*M* ammonium acetate. However, their size and quality were not suitable for X-ray data collection. Further optimization of the crystallization conditions was achieved by screening addi-

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shells (2.64–2.50 Å for native data and 2.28–2.20 Å for SeMet data).

Data	Native	SeMet
X-ray wavelength (Å)	0.9500 (PF 18B)	0.9792 (PLS 6B)
Temperature (K)	100	100
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 48.64, b = 83.13, c = 117.33	a = 48.42, b = 82.61, c = 115.71
Resolution range (Å)	30.0–2.50	30.0–2.20
Total/unique reflections	150 357/15 313	396 736/24 031
$R_{\rm merge}$† (%)	7.7 (33.2)	5.1 (13.3)
Data completeness (%)	88.8 (88.8)	93.9 (86.6)
Average $I/\sigma(I)$	8.2 (2.3)	44.7 (11.0)

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

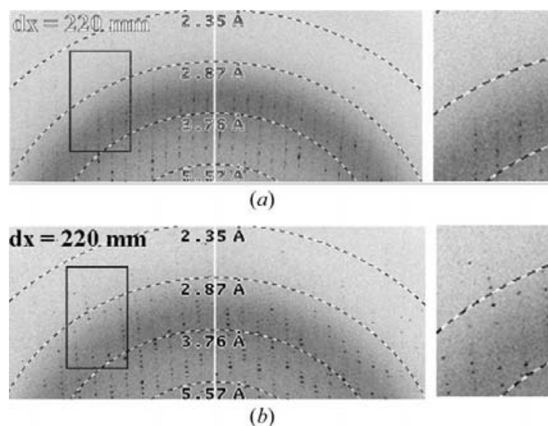

Figure 2
A comparison of X-ray diffraction images (*a*) before and (*b*) after dehydration/annealing of a native crystal. This technique resulted in a significant improvement of the diffraction limit and the mosaicity. The crystal-to-detector distance, dx, was 220 mm. A magnified view of a boxed region is shown on the right.

tives. Better crystals were obtained when ethylene glycol was added to the hanging drop, its final concentration being adjusted to 3.0%(v/v). Rectangular crystals grew to dimensions of 0.4 × 0.3 × 0.15 mm within a week (Fig. 1). They were reasonably stable for mechanical treatment and X-ray exposure.

Since the flash-frozen crystals diffracted to ~3.0 Å and their spot shapes were elongated, we attempted crystal dehydration/annealing, which significantly improved the diffraction quality. The diffraction pattern extended to ~2.5 Å and the mosaicity became smaller (Fig. 2). It is conceivable that the protein molecules in the crystal lattice slightly rearranged themselves during crystal dehydration/annealing and the crystal became better ordered. A total of 15 313 unique reflections were measured with a redundancy of 9.8, with a completeness of 88.8% and an $R_{\rm merge}$ of 7.7%. The space group was determined to be $P2_12_12_1$ from the systematic absences of reflections; the unit-cell parameters were a = 48.6, b = 83.1, c = 117.3 Å, $\alpha = \beta = \gamma = 90°$. The unit-cell parameters were fixed for processing the data. Table 1 summarizes the statistics of data collection. The presence of two monomers of the recombinant CKS in the crystallographic asymmetric unit gives a crystal volume per protein mass ($V_{\rm M}$) of 2.05 Å^3 Da^{-1}, with a solvent content of 40.0% (Matthews, 1968).

Employing the model of *E. coli* K-CKS (PDB code 1he7; Jelakovic & Schulz, 2001) as a probe, molecular-replacement trials gave a solution which could not readily be refined to a reasonably low R value. Therefore, we prepared a SeMet-substituted protein and crystallized it under conditions identical to those for the native crystal. We found that the SeMet crystals diffracted to a higher resolution even without crystal dehydration/annealing. Owing to a shortage of synchrotron beam time, however, we only collected X-ray data to 2.2 Å at a single wavelength (Table 1). The unit-cell parameters are a = 48.42 (1), b = 82.61 (12), c = 115.71 (17) Å, where the estimated standard deviations are given in parentheses. Single-wavelength data were not sufficient for phasing. In order to solve the structure by the multi-wavelength anomalous diffraction (MAD) method, we plan to collect MAD data at three different wavelengths when sufficient synchrotron beam time becomes available.

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