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Preliminary X-ray analysis of a new crystal form of the *Escherichia coli* KDO8P synthase

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3-Deoxy-D-*manno*-octulosonate 8-phosphate (KDO8P) synthase catalyzes the biosynthesis of an essential component of the lipopolysaccharide of all Gram-negative bacteria. The structure and mechanism of KDO8P synthase are being actively studied as this enzyme represents an important target for antibiotic therapy. The structure of the *Escherichia coli* KDO8P synthase in cubic crystals (space group $I23$) has recently been determined and the enzyme shown to be a tetramer of identical subunits. However, this information is challenged by biochemical studies, which suggest that the enzyme behaves in solution as a homotrimer. Here, the preparation and preliminary X-ray analysis of monoclinic crystals of KDO8P synthase are reported. The crystals belong to space group $P2_1$, with unit-cell parameters $a \simeq 50$, $b \simeq 140$, $c \simeq 74$ Å, $\beta \simeq 105^\circ$. The structure of KDO8P synthase in the monoclinic crystal form was determined by molecular replacement, using as a search model one of the subunits of the enzyme in the cubic crystals. A tetramer of KDO8P synthase with 222 local symmetry is also present in the asymmetric unit of the $P2_1$ crystals, with a solvent content of 43%. The observation that the same quaternary structure of KDO8P synthase is observed in two different crystal forms belonging to distinct crystal systems (monoclinic and cubic) suggests that a tetramer is the native form of the enzyme.

1. Introduction

3-Deoxy-D-*manno*-octulosonate 8-phosphate (KDO8P) synthase (E.C. 4.1.2.16) catalyzes the condensation of phospho*enol*pyruvate and arabinose 5-phosphate to form KDO8P and inorganic phosphate. KDO8P is the phosphorylated precursor of 3-deoxy-D-*manno*-octulosonate (KDO), an eight-carbon sugar that constitutes an essential part of the lipopolysaccharide (LPS) of Gram-negative bacteria (Raetz, 1990). The LPS biosynthetic pathway is an attractive target for multi-drug therapy, since mutants that produce incomplete LPS are both less pathogenic and more susceptible to known antibiotics (Kropinski *et al.*, 1978; Hiruma *et al.*, 1984; Hammond, 1992; Vaara, 1993). In particular, the biosynthetic pathway for KDO is an excellent candidate for the development of new therapeutic agents that may be more globally effective as antibiotics, as KDO is a sugar found exclusively in Gram-negative bacteria and not in mammalian cells.

The *E. coli* KDO8P synthase is encoded by the *kdsA* gene (Woisetschlager & Hogenauer, 1986). The translated product contains 284 amino acids with a calculated M_r of 30 833 Da. Early studies of KDO8P synthase suggested that the enzyme is a trimer of identical subunits (Ray, 1980; Woodard, unpublished results). Furthermore, two different crystal forms of the *E. coli* KDO8P synthase have been reported (Tolbert *et al.*, 1996), both belonging to cubic space groups. These preliminary crystallographic studies have been interpreted as additional evidence that KDO8P synthase is a homotrimer and that its inclusion in a three-dimensional lattice exploits the coincidence between a crystallographic and a local threefold axis of symmetry. We have recently determined the structure of the *E. coli* KDO8P synthase (Radaev *et al.*, 2000) in cubic crystals that, based on the space group and unit-cell parameters ($I23$, a = 228.6 Å), appear to be essentially identical to the cubic crystals of the B form described by Tolbert *et al.* (1996). Surprisingly, in these crystals one tetramer of KDO8P synthase occupies the asymmetric unit. Furthermore, no sensible arrangement of subunits is observed around the crystallographic threefold axes that would support a trimeric architecture of the enzyme. The difference in the predicted quaternary structure of the enzyme as determined by chromatographic and crystallographic methods could be interpreted as arising from either an anomalous migration of a tetrameric enzyme during molecular-sieve fractionation or to a crystallization artifact. In order to resolve this

controversy, we have pursued the study of a different crystal form of KDO8P synthase, in the hope that it might provide an independent verification of the enzyme quaternary structure. Here, we report the crystallization and structure determination of the *E. coli* KDO8P synthase in a monoclinic space group and show that a homotetramer of the enzyme also occupies the asymmetric unit of these new crystals.

2. Results and discussion

2.1. Crystallization and X-ray data collection

Crystals of *E. coli* KDO8P synthase were obtained by vapor diffusion in hanging drops at 296 K. Drops (15 µl) containing 10 mg ml^{-1} KDO8P synthase, 20% saturated ammonium sulfate, 50 m*M* Na HEPES pH 7.4, 3%(*v*/*v*) ethylene glycol, 3 m*M* octyl-D-*pyrano*-glucoside were equilibrated against a reservoir containing 1 ml 40% saturated ammonium sulfate, 100 m*M* Na HEPES pH 7.4, 5%(*v*/*v*) ethylene glycol. Rod-shaped crystals of KDO8P synthase with average dimensions of 0.1 × 0.1 × 0.4 mm appeared after 2–3 weeks. These crystals belong to space group $P2_1$, with unit-cell parameters $a \simeq 50$, $b \simeq 140$, $c \simeq 74$ Å, $\beta \simeq 105^\circ$ (Table 1), and can be stored indefinitely in a cryoprotectant holding solution consisting of 60% saturated ammonium sulfate, 50 m*M* Na HEPES pH 7.4, 20%(*v*/*v*) ethylene glycol, 3 m*M* octyl-D-*pyrano*-glucoside.

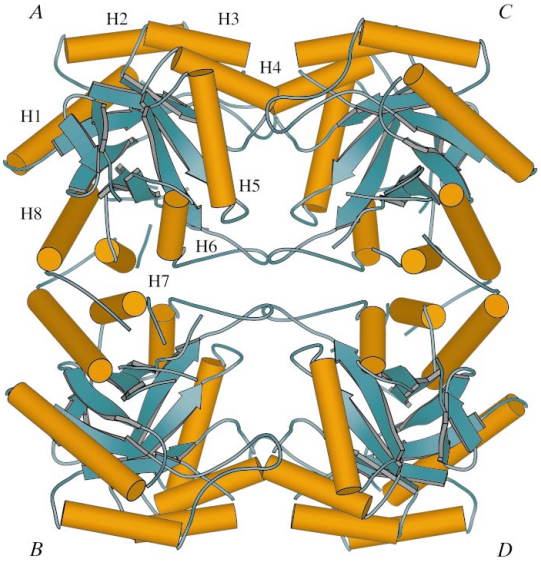

Figure 1
Structure of KDO8P synthase. Cartoon of the tetrameric enzyme looking down one of the twofold axes of local symmetry. Strands are represented as arrows and helices as cylinders. H, helix. This figure was generated with *MOLSCRIPT* (Kraulis, 1991).

2.2. Structure determination and refinement

X-ray diffraction data at the resolution limit of 3.2 Å were collected from a single crystal flash-frozen at 100 K on a Rigaku rotating-anode generator equipped with a graphite monochromator using a Bruker Hi-Star area detector. Oscillation data were processed with *X-GEN* (Biosym Inc.). Structure determination by molecular replacement and crystallographic refinement was carried out using *CNS* v0.9 (Brunger *et al.*, 1998). A real-space self-rotation function analysis of the experimental data set in the resolution range 15–4 Å was performed in the rotational intervals $\psi = 0$–180°, $\varphi = 0$–180°, $\kappa = 0$–180°. A cluster analysis of the output of this search did not reveal the presence of local axes of symmetry. Additionally, only peaks originating from crystallographic symmetry appeared when the self-rotation function analysis was displayed as a three-dimensional map contoured at 2σ (not shown). This result was somewhat surprising, because the presence of only one chain of KDO8P synthase in the asymmetric unit of the monoclinic crystal would correspond to a solvent content of 75% and also to a markedly different quaternary structure of the enzyme compared with that observed in the cubic crystals of KDO8P synthase. A real-space cross-rotation function search using as a model one of the chains of the tetramer from the cubic crystal structure (Radaev *et al.*, 2000) produced two solutions at $\sim 2\sigma$ above the mean value of the function, two at $\sim 1.5\sigma$ and a series of solutions at $\sim 1.2\sigma$. As the absence of solutions clearly above the background could be a consequence of a high noise level in the real-space rotation function, a direct cross-rotation function search (Brünger, 1997) was performed in the 15–4 Å resolution range. For this purpose the 'fast direct' implementation of the direct rotation function of *CNS* v0.9 was adopted: this consisted of an initial search using a coarse grid of 13° angular size, followed by analysis of the top 20 peaks on a finer grid of 2.6° angular size. This search produced four distinct solutions clearly above the background (>3.5σ), suggesting that a tetramer might be present also in the asymmetric unit of the monoclinic crystal form of KDO8P synthase. Four sequential translation searches were carried out to identify the relative positions of the monomers. The final translation placed a complete tetramer of KDO8P synthase in the asymmetric unit. This solution was refined first by means of a rigid-body minimization in which each chain was treated as an independent group and then by torsion-angle dynamics using a slow-cooling protocol from 1000 to 300 K. Finally, conjugate-gradient minimization of the positional parameters was followed by refinement of two group *B* factors (one for the backbone and one for the side chain) for each residue. NCS restraints were implemented throughout the refinement, which was carried out using cross-validated maximum likelihood (Adams *et al.*, 1997) as the target function. The current *R* and $R_{\rm free}$ factors, prior to any fine tuning of the model by manual intervention, are 24 and 29%, respectively. Data-collection and refinement statistics are reported in Table 1. An *a posteriori* analysis revealed that the same solution could also be obtained in a more straightforward way using the entire tetramer of KDO8P synthase from the cubic crystals as the search model.

Table 1
Data-collection and refinement statistics.

This data set was collected with only one crystal flash-frozen at 100 K. Numbers in parentheses refer to the highest resolution shell.

Unit-cell parameters	
a (Å)	50.2
b (Å)	140.2
c (Å)	74.2
β (°)	104.9
Space group	$P2_1$
Data-collection device	Bruker Hi-Star
Resolution (Å)	32–3.2 (3.4–3.2)
Number of unique observations	15871 (2339)
Completeness	96.8 (95.1)
Redundancy	3.9 (3.1)
$R_{\rm merge}$†	0.11 (0.36)
$\langle I/\sigma(I)\rangle$	5.2 (2.3)
$R_{\rm cryst}$‡	0.24 (0.32)
$R_{\rm free}$§	0.29 (0.38)

† $R_{\rm merge} = \sum_h \sum_i |I_{h,i} - \langle I_h\rangle| / \sum_h \sum_i |I_{h,i}|$, where $\langle I_h\rangle$ is the mean intensity of the i observations of reflection h. ‡ $R_{\rm cryst} = \sum_h |F_o - F_c| / \sum_h |F_o|$, where F_o and F_c denote the observed and calculated structure-factor amplitudes, respectively. § $R_{\rm free}$ was calculated on 10% of the data.

2.3. Quaternary structure of KDO8P synthase

The structure of KDO8P synthase in monoclinic crystals is almost identical to that

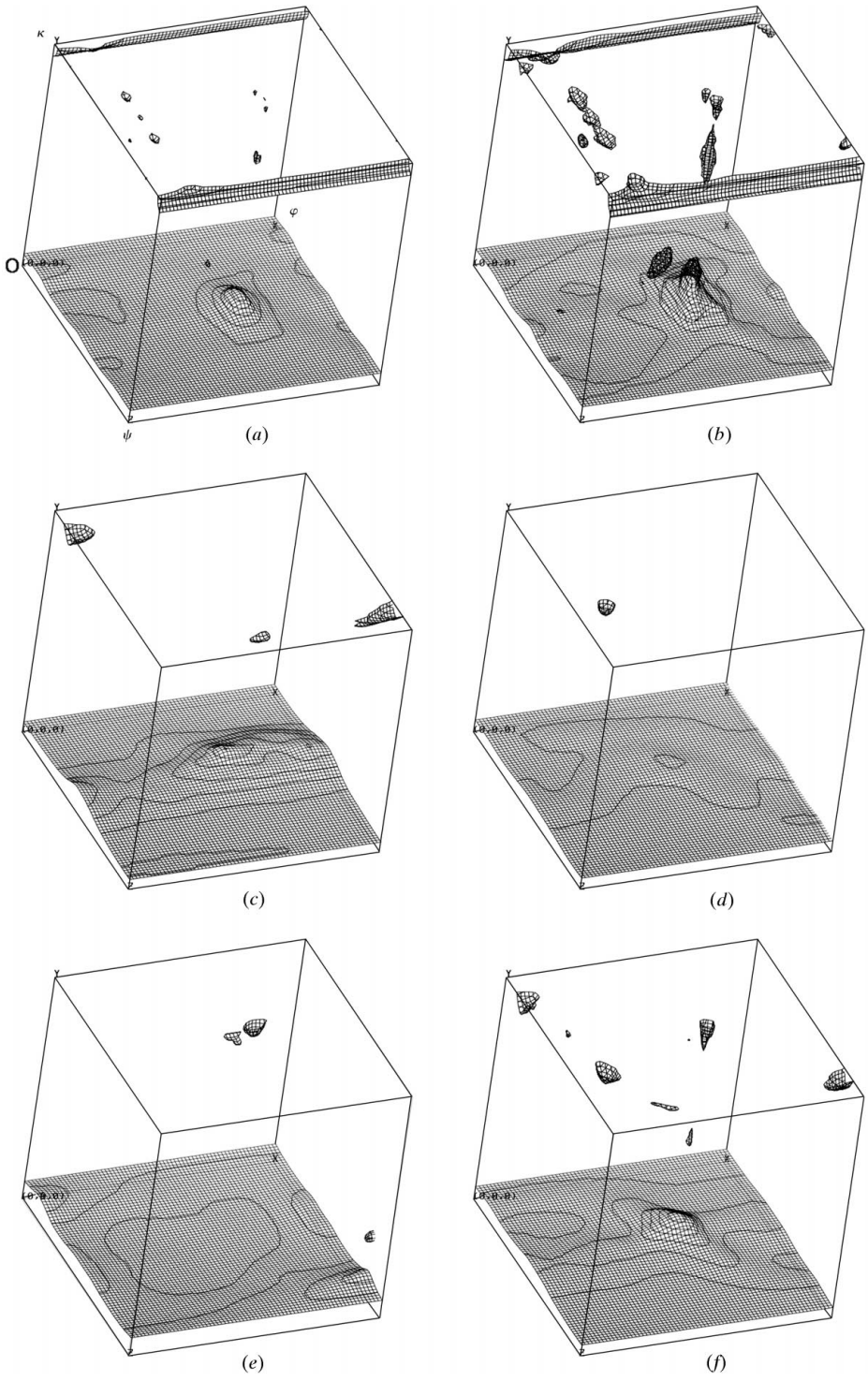

Figure 2
Self-rotation function analyses of experimental and calculated data sets in the range $\varphi = \psi = \kappa = 0$–$180^\circ$ are represented as three-dimensional maps covering cubes whose axes X, Y and Z correspond to φ, κ and ψ, respectively. (*a*) Experimental data set (self-rotation function contoured at 1σ): only the crystallographic twofold axis ($\psi = 0$ or 180, $\kappa = 180$, $\varphi = 0$–180°) is clearly visible. (*b*) The same as (*a*) but contoured at 0.5σ to show the additional peaks in the $\kappa = 180^\circ$ plane originating from the twofold axes of the two tetramers contained in the unit cell. (*c*) Data set calculated from subunits *A* and *B* of the tetramer placed in a *P*1 cell (contoured at 1σ): two equivalent peaks are visible corresponding to $\psi = 25.5$ or 154.5, $\varphi = 0$ or 180, $\kappa = 180^\circ$. (*d*) Data set calculated from subunits *A* and *C* of the tetramer in a *P*1 cell (contoured at 1σ): a single peak is visible at $\psi = 113.3$, $\varphi = 23.3$, $\kappa = 180^\circ$. (*e*) Data set calculated from subunits *A* and *D* of the tetramer in a *P*1 cell (contoured at 1σ): a single peak is visible at $\psi = 80.6$, $\varphi = 109.2$, $\kappa = 180^\circ$. (*f*) Data set calculated from an entire tetramer of KDO8P synthase placed in a *P*1 cell: all the peaks observed in (*c*), (*d*) and (*e*) are now present. Notice the correspondence between these peaks (originating from one tetramer) and half of those derived from the analysis of the experimental data set (*a*, *b*), which contains information on both tetramers present in the unit cell.

of the enzyme in cubic crystals (space group $I23$, $a = 228.6$ Å) reported by Radaev *et al.* (2000). As previously mentioned, these cubic crystals probably correspond to the crystal form B described by Tolbert *et al.* (1996). A homotetramer (subunits *ABCD*) with 222 local symmetry occupies the asymmetric unit of the monoclinic crystal (Fig. 1); each monomer has the fold of a typical $(\beta/\alpha)_8$ barrel (eight-stranded parallel β-barrel surrounded by eight helices). While the cubic crystals of KDO8P synthase are mostly occupied by solvent (70% solvent content) such that only subunits *A*, *B* and *C* are involved in crystal contacts, the lattice of the monoclinic crystals of KDO8P synthase is significantly more populated (43% solvent content) and all the subunits of the enzyme are involved in crystal contacts.

Although earlier biochemical studies (Ray, 1980) have provided evidence that KDO8P synthase from *E. coli* is a trimer of identical subunits, the observation that a homotetramer occupies the asymmetric unit of two different crystal forms belonging to distinct crystal systems (monoclinic and cubic) strongly suggests that the tetramer represents the native form of the enzyme.

It is of general interest to understand the reasons for the failure of the self-rotation function analysis to identify correctly the presence of a tetramer in the asymmetric unit of the monoclinic crystal. One possible explanation is that peaks corresponding to the twofold axes of the tetramer can be detected only at very low levels of signal-to-noise ratio. Indeed, additional peaks not originating from the space-group symmetry appear when the self-rotation function analysis is displayed as a three-dimensional map contoured at 1.0 and 0.5σ, respectively (Figs. 2*a* and 2*b*). These peaks can be compared with the peaks obtained by performing a self-rotation function analysis using amplitudes calculated by placing only one tetramer or different pairs of monomers (in the positions derived from the refined structure) in a *P*1 cell identical in dimensions to the unit cell of the monoclinic crystals of KDO8P synthase. A single peak is produced when the subunit pairs *AB*, *AC* or *AD* are used in the calculations, each corresponding to one of the three twofold axes of symmetry of the enzyme (Figs. 2*c*, 2*d* and 2*e*). Three peaks are produced when the entire tetramer is used (Fig. 2*f*). The

remaining peaks present in the self-rotation function analysis of the experimentally derived amplitudes originate from the second tetramer present in the unit cell of the monoclinic crystals. These observations suggest that the correct derivation of the arrangement of subunits in the asymmetric unit of a crystal solely from self-rotation function analysis may require at times the consideration of extremely weak signals. It is also of note that, while the correct orientations of the four subunits of KDO8P synthase could be easily identified as the four highest peaks (>3.5σ) in a direct cross-rotation function search, the same four orientations appeared only as peaks at 1.2–1.5σ above the mean in a real-space cross-rotation function search. Thus, the data reported here provide additional evidence that direct rotation-function searches are more sensitive than their real-space counterparts (Brünger, 1997).

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