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Structure of 2-keto-3-deoxy-D-manno-octulosonate-8-phosphate synthase from *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a major cause of opportunistic infection and is resistant to most antibiotics. As part of efforts to generate much-needed new antibiotics, structural studies of enzymes that are critical for the virulence of *P. aeruginosa* but are absent in mammals have been initiated. 2-Keto-3-deoxy-D-manno-octulosonate-8-phosphate synthase (KDO8Ps), also known as 2-dehydro-3-deoxyphosphooctonate aldolase, is vital for the survival and virulence of *P. aeruginosa*. This enzyme catalyzes a key step in the synthesis of the lipopolysaccharide (LPS) of most Gram-negative bacteria: the condensation reaction between phosphoenolpyruvate (PEP) and arabinose 5-phosphate to produce 2-keto-3-deoxy-D-manno-octulosonate-8-phosphate (KDO8P). This step is vital for the proper synthesis and assembly of LPS and the survival of *P. aeruginosa*. Here, the recombinant expression, purification and crystal structure of KDO8Ps from *P. aeruginosa* are presented. Orthorhombic crystals were obtained by vapor diffusion in sitting drops in the presence of 1 mM phosphoenolpyruvate. The structure reveals the prototypical α/β TIM-barrel structure expected from this family of enzymes and contains a tetramer in the asymmetric unit.

1. Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is a major cause of opportunistic infections and is resistant to many antibiotics and disinfectants. *P. aeruginosa* is capable of growing in a variety of habitats, including soil, marshes and coastal marine habitats, and has even been found thriving in tarballs from oil spills in Nigeria (Itah & Essien, 2005). *P. aeruginosa* also grows on plants and animal tissue, such as the lungs of cystic fibrosis (CF) patients, and has the ability to form biofilms on medical devices (Gaspar *et al.*, 2013). *P. aeruginosa* infection is a primary cause of mortality of CF patients since infections are often impossible to eradicate owing to the natural resistance of *P. aeruginosa* to antibiotics (Olivares *et al.*, 2013). *P. aeruginosa* is also a primary source of hospital-acquired infections, including bacteremia in burn victims and urinary-tract infections in catheterized patients, as well as pneumonia in patients on ventilators and respirators (Chastre & Fagon, 2002; Fagon, Chastre, Hance, Montravers *et al.*, 1993; Fagon, Chastre, Hance, Domart *et al.*, 1993; Fagon, Chastre, Domart *et al.*, 1996; Fagon, Trouillet *et al.*, 1996; Fagon & Chastre, 2000; Fagon, Chastre, Vuagnat *et al.*, 1996; Fagon *et al.*, 2000). Owing to its resistance to conventional antibiotics, there is a need to develop new therapeutics to reduce the morbidity and mortality of *P. aeruginosa* infections. An alternative strategy for therapeutic intervention is to target enzymes implicated in pathways that are unique to *P. aeruginosa* and are not found in humans.

One such enzyme, 2-keto-3-deoxy-D-manno-octulosonate-8-phosphate synthase (KDO8Ps), also known as 2-dehydro-3-deoxyphosphooctonate aldolase, is vital for the survival and virulence of most Gram-negative bacteria. The enzyme KDO8Ps catalyzes the condensation reaction between phosphoenolpyruvate (PEP) and arabinose 5-phosphate (A5P) to produce 2-keto-3-deoxy-D-manno-octulosonate-8-phosphate (KDO8P). This reaction is essential to the assembly of lipopolysaccharide (LPS) in *P. aeruginosa* and other

Gram-negative bacteria and is consequently an attractive target for therapeutic intervention (Asojo *et al.*, 2001). Here, we present the crystal structure of KDO8Ps from *P. aeruginosa* (*Pa*KDO8Ps). These studies are part of our ongoing structure-based therapeutic discovery studies for drug-resistant bacteria. This project was also used as a practicum in protein crystallography for summer pre-baccalaureate researchers.

2. Materials and methods

2.1. Expression and purification

The *kdo8ps* gene of *P. aeruginosa* strain PAO1 (NCBI reference sequence NP_252326.1) was used as a template for synthesis of a DNA expression construct encoding the 289-amino-acid protein fused to an N-terminal hexahistidine tag (GenScript USA Inc., Piscataway, New Jersey, USA). This synthetic construct was codon-optimized for expression in *Escherichia coli* and was cloned into the *Nde*I/*Not*I sites of pET28a vector (Novagen, Madison, Wisconsin, USA). The resulting plasmid was transformed into *E. coli* strain Rosetta2 (pLysS) DE3 by heat shock. Positive colonies were identi-

fied by colony PCR with T7 promoter and T7 terminator primers (EMD Millipore, Rockland, Massachusetts, USA). Initial small-scale expression screens were performed on ten positive clones by culturing a single colony in 10 ml NZYM broth containing $50 \mu\text{g ml}^{-1}$ kanamycin to an OD_{600} of 0.7. Protein induction was initiated by the addition of IPTG to a final concentration of 0.5 mM for 16 h at 301 K. The highest expressing clone was selected for large-scale expression in 1.2 l cultures using a 2.8 l Fernbach flask. The cell pellets were harvested by centrifugation, suspended in lysis buffer (100 mM Tris-HCl pH 7.5 with 5% Triton X-100, 10% glycerine and Roche complete EDTA-free protease-inhibitor cocktail) and lysed under high pressure in an Emulsiflex homogenizer (Avestin Canada). The protein in the clarified supernatant was purified by Ni-affinity chromatography on a Ni FF crude column (GE Healthcare, Piscataway, New Jersey, USA) using 100 mM Tris pH 8.0, 150 mM NaCl and was eluted with 250 mM imidazole. The typical total yield of pure protein was 60 mg from 1.2 l of culture. Coomassie-stained SDS-PAGE was used to assess the purity of the eluted protein fractions (Fig. 1). The fractions with greater than 95% purity (A_7 – A_{10}) were combined, dialyzed into storage buffer (50 mM Tris pH 7.5, 0.05% β -mercaptoethanol) and stored at 193 K prior to use in crystallization studies. The other fractions were combined and further purified by Ni-affinity chromatography.

2.2. Crystallization

The protein was concentrated to 5 mg ml^{-1} in buffer composed of 0.2 M Tris-HCl pH 7.4, 0.1 M NaCl, 0.05% β -mercaptoethanol, 0.1 mM PEP prior to setting up crystal screens. Crystals were grown at 293 K by vapor diffusion in sitting drops. Drops were prepared by mixing 3 μl protein solution with 1.5 μl reservoir solution. The reservoir solution consisted of 0.2 M calcium acetate, 0.1 M sodium

Figure 1
Protein purity as assessed by Coomassie Blue-stained SDS-PAGE. The fractions eluted from the affinity column are greater than 95% pure. The mass of the protein is close to the theoretically calculated 32 kDa. Lanes A_2 – A_{10} contain eluted fractions, while lane M contains marker (labelled in kDa) and lane FT is the flowthrough.

Figure 2
*Pa*KDO8Ps crystals in a drop. The long, thin and flat plate-like rod-shaped crystals were smaller than $0.05 \mu\text{m}$ on the smallest side and up to 1.0 mm on the longest side.

Figure 3
*Pa*KDO8Ps structure. Ribbon diagram of the homotetramer of *Pa*KDO8Ps with monomer A in blue, monomer B in magenta, monomer C in green and monomer D in orange. Each monomer reveals the characteristic α/β TIM-barrel fold. Approximately $190 \text{ \AA}^2$ of surface area is buried at the interface of monomers A with B as well as of C with D. The buried surface for the interaction of monomers A with D and of C with B is $1300 \text{ \AA}^2$. The interface for the interaction of monomers A with C and of B with D is approximately $1000 \text{ \AA}^2$.

cacodylate pH 6.5, 18%(w/v) polyethylene glycol 8000. Small clear crystals of less than 0.05 mm on the smallest face were obtained within 2 d (Fig. 2). A single flat rod-shaped crystal of approximate dimensions $0.05 \times 0.1 \times 0.6$ mm was flash-cooled directly in a stream of N_2 prior to data collection at 100 K.

2.3. Data collection and structure determination

X-ray diffraction data were collected at the Baylor College of Medicine core facility using a Rigaku HTC detector and a Rigaku FR-E+ SuperBright microfocus rotating-anode generator with VariMax HF optics. A data set was collected from a single crystal with a crystal-to-detector distance of 115 mm and an exposure time of 120 s for 0.5° oscillations using the *CrystalClear* (*d*TREK*) package (Pflugrath, 1999). Data were processed using *MOSFLM* (Leslie, 2006). The crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 79.43$, $b = 94.85$, $c = 145.72$ Å. The structure was solved by molecular replacement with *Phaser* (McCoy *et al.*, 2005; Storoni *et al.*, 2004). The molecular-replacement search model was a monomer of KDO8Ps from *E. coli* (PDB entry 1g7v)

stripped of all waters and ligands (Asojo *et al.*, 2001). Molecular replacement was followed by iterative cycles of model building with *Coot* (Emsley *et al.*, 2010) and structure refinement with *REFMAC5* (Murshudov *et al.*, 2011) within the *CCP4* package (Winn *et al.*, 2011). Unless otherwise noted, figures were generated using *PyMOL* (DeLano, 2002).

3. Results and discussion

3.1. Overall structure

The refined model of *PaKDO8Ps* has four monomers in the asymmetric unit and each monomer has the overall α/β -barrel structure reported for homologous enzymes (Fig. 3). The structure has the overall topology of an aldolase-type TIM barrel. Only 247 amino acids are ordered in each monomer and these residues are comprised of 35.2% α -helices, 4.5% 3_{10} -helices, 21.1% strands and 39.3% loop regions. The disordered residues are in the N-terminal hexahistidine tag and the C-termini as well as in two C-terminal regions that are in proximity to the KDO8Ps active site. The two

Figure 4

Two-dimensional structural alignment of *PaKDO8Ps* with homologous KDO8Ps. Sequence and structural alignment with the closest structural homologues reveals a conserved overall structure. The compared sequences are *NmKDO8Ps* (3-deoxy-D-manno-octulosonate-8-phosphate synthase from *N. meningitidis*; PDB entry 3ste; Allison *et al.*, 2011), *EcKDO8Ps* (from *E. coli*; PDB entry 1g7v; Asojo *et al.*, 2001), *BpKDO8Ps* (from *B. pseudomallei*; PDB entry 3und; Seattle Structural Genomics Center for Infectious Disease, unpublished work) and *AaKDO8Ps* (homolog from *A. aeolicus*; PDB entry 1fiwt; Ackerman & Gatti, 2011). This figure was generated with *ESPrPr* (Gouet *et al.*, 1999, 2003).

disordered C-terminal regions are Met210–Gly220 and Pro246–Leu261.

The four monomers in the asymmetric unit superpose well, with r.m.s. deviations of 0.50, 0.638 and 0.57 Å for all main-chain atoms for chains *B*, *C* and *D* compared with chain *A*. There is extensive buried surface area in the *Pa*KDO8Ps homotetramer. Each monomer forms two major dimer interactions and one minor dimer interaction with the other three monomers (Fig. 3). The two major interfaces are approximately 1000 and 1300 Å², while the minor interface is only approximately 190 Å². The major dimer interfaces could potentially be much larger since they are in close proximity to both disordered C-terminal loop regions. Details of the quality of the structure as well

as data collection are shown in Table 1. The atomic coordinates and structure factors have been deposited in the PDB as entry 4lu0.

3.2. Comparison to other KDO8Ps

The structures that are most similar to that of *Pa*KDO8Ps were identified using the Structure Similarity option in *PDBFold* (<http://www.ebi.ac.uk/msd-srv/ssm/>). The highest structural similarity was found for the crystal structures of mutants of *Neisseria meningitidis* 3-deoxy-D-manno-octulosonate-8-phosphate synthase (*Nm*KDO8Ps; Allison *et al.*, 2011), which shares 68% sequence identity with *Pa*KDO8Ps. Other known structures that share considerable sequence identity with *Pa*KDO8Ps include the homologous enzymes from *E. coli* (PDB entry 1g7v; Asojo *et al.*, 2001), with 69% sequence similarity, *Burkholderia pseudomallei* (PDB entry 3und; Seattle Structural Genomics Center for Infectious Disease, unpublished work), with 64% sequence identity, and *Aquifex aeolicus* (PDB entry 1fwf; Ackerman & Gatti, 2011), with 46% sequence identity. The monomers of *Pa*KDO8Ps superpose quite well with those from the homologous enzymes, with an r.m.s. deviation of around 1.2 Å for all main-chain atoms of the models. The regions of highest structural variability for these proteins tend to be in loop regions, most notably those in proximity to the putative active site (Figs. 4 and 5). The functional unit of KDO8Ps is believed to be a tetramer and *Pa*KDO8Ps superposes well with the homotetramer of the structure of *Ec*KDO8Ps (PDB entry 1d9e; Radaev *et al.*, 2000), with an r.m.s. deviation of 0.70 Å for all main-chain atoms. Similar tetramers can be generated across the crystallographic symmetry of homologous KDO8Ps.

3.3. Active site of *Pa*KDO8Ps

There are two classes of KDO8Ps: those that require a metal for catalysis and those that do not (Allison *et al.*, 2011; Tao *et al.*, 2010). Representatives of both classes of KDO8Ps have been structurally

Figure 5

Comparison of the *Pa*KDO8Ps structure with homologous structures. (a) The superposed monomers of *Pa*KDO8Ps align well with the structural homolog *Nm*KDO8Ps, with highest variation in the loop regions. (b) *Pa*KDO8Ps is structurally very similar to the structure of the ternary complex of *Aa*KDO8Ps with PEP and A5P. (c) The structure of *Ec*KDO8Ps with an inhibitor is also very similar to that of *Pa*KDO8Ps. The main differences between all of the structures are in the loop regions and especially those in proximity to the large active-site region. The monomers of *Pa*KDO8P synthase are colored as follows: monomer *A* in blue, monomer *B* in magenta, monomer *C* in green and monomer *D* in orange. *Ec*KDO8P (PDB entry 1g7v) is colored gray and *Nm*KDO8P (PDB entry 3ste) is colored aquamarine, while *Aa*KDO8Ps (PDB entry 1fwf) is colored tan.

Table 1Crystallographic data-collection and refinement statistics for *PaKDO8Ps*.

Values in parentheses are for the highest resolution shell.

Unit-cell parameters	$a = 79.27, b = 95.1, c = 145.14$
Space group	$P2_12_12_1$
Resolution (Å)	60.9–2.8 (2.9–2.8)
$R_{\text{merge}}^{\dagger}$ (%)	18.5 (51.0)
Completeness (%)	96.5 (76.8)
Multiplicity	5.9 (2.8)
$\langle I/\sigma(I) \rangle$	7.1 (1.5)
Refinement	
R factor ‡ (%)	20.6 (29.0)
$R_{\text{free}}^{\S}$ (%)	25.9 (32.4)
Correlation coefficients	
$F_o - F_c$	0.938
$F_o - F_c, \text{ free}$	0.885
Components of model	
Amino-acid residues	999
Waters	40
Mean B factor (Å ²)	49.3
R.m.s. deviation from ideal	
Bond lengths (Å)	0.011
Bond angles (°)	1.635
Chirality (Å ³)	0.105
Ramachandran plot, residues in (%)	
Favored regions	97.6
Allowed regions	1.23
Outlier regions	1.13

$^{\dagger} R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity obtained from multiple observations of symmetry-related reflections after rejections. $^{\ddagger} R$ factor = $\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} are observed and F_{calc} are calculated structure factors. § The R_{free} set was a randomly chosen 5% of the reflections.

characterized and reveal large central cavities consisting of PEP-binding and A5P-binding regions (Kona *et al.*, 2009; Ackerman & Gatti, 2011; Asojo *et al.*, 2001). Unsurprisingly, our structure of *PaKDO8Ps* has a large active site that consists of binding sites for PEP and A5P. Although the substrate PEP was added to the protein at 1 mM prior to crystallization, no density was observed for PEP and essentially our structure appears to be that of the unbound enzyme. The helices and strands of *PaKDO8Ps* superpose well with the structures of homologous enzymes with bound inhibitor or substrates (Fig. 5). The loop regions in proximity to the active site are known to have conformational flexibility and these affect substrate binding. Thus, we need to determine structures of the complex with inhibitors and substrates in order to unambiguously clarify the catalysis process of *PaKDO8Ps*.

4. Concluding remarks

PaKDO8Ps crystallized with a tetramer in the asymmetric unit. The tertiary and quaternary structures of *KDO8Ps* are very similar to those of homologous enzymes. Our structure of *PaKDO8Ps* reveals features that suggest that *PaKDO8Ps* will be inhibited by similar inhibitors as homologous enzymes. However, efforts at rational drug design will have to take into account the conformational flexibility of the loops in proximity to the active sites. Future studies include cocrystallization studies with substrates, metals and inhibitors in order to define how these affect the binding cavity of *PaKDO8Ps*.

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