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Structure of 3-deoxy-*manno*-octulosonate cytidylyltransferase from *Haemophilus influenzae* complexed with the substrate 3-deoxy-*manno*-octulosonate in the β-configuration

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The enzyme 3-deoxy-*manno*-octulosonate cytidylyltransferase (CMP-KDO synthetase; CKS) catalyzes the activation of 3-deoxy-D-*manno*-octulosonate (or 2-keto-3-deoxy-*manno*-octonic acid; KDO) by forming CMP-KDO. CKS is unique to Gram-negative bacteria and is an attractive target for the development of antibacterial agents. The crystal structure of CKS from *Haemophilus influenzae* in complex with the substrate KDO has been determined at 2.30 Å resolution by combining single-wavelength anomalous diffraction and molecular-replacement methods. The two monomers in the asymmetric unit differ in the conformation of their C-terminal α-helix (Ala230–Asn254). The KDO bound to the active site exists as the β-pyranose form in the $^5C_2$ chair conformation. The structure of CKS from *H. influenzae* in complex with KDO will be useful in structure-based inhibitor design.

1. Introduction

3-Deoxy-*manno*-octulosonate (or 2-keto-3-deoxy-*manno*-octonic acid; KDO) is an essential component of lipopolysaccharides (LPS) in Gram-negative bacteria. Incorporation of KDO into LPS requires its activation by 3-deoxy-*manno*-octulosonate cytidylyltransferase (CMP-KDO synthetase; CKS). Two isozymes of CKS have been found in *Escherichia coli* (Jelakovic & Schulz, 2002). One of them, named L-CKS, is encoded by the *kdsB* gene and participates in the biosynthesis of LPS. The other isozyme, K-CKS, is encoded by the *kpsU* gene and is involved in capsule expression (Rosenow *et al.*, 1995). CKS is unique to Gram-negative bacteria and is absent from mammalian cells (Jelakovic & Schulz, 2001). Therefore, CKS is an attractive target for the development of antibacterial agents (Goldman *et al.*, 1987).

Three-dimensional structures of K-CKS from *E. coli* [apo, PDB codes 1h6j and 1h7e; cytidine-5′-monophosphate (C5P) complex, 1h7f; cytidine-5′-triphosphate (CTP) complex, 1h7g and 1gq9; cytidine-5′-diphosphate complex, 1h7h; C5P and *O*-sialic acid complex, 1h7t; cytidine-5′-monophosphate-3-deoxy-β-D-*gulo*-oct-2-ulo-pyranosonic acid (CMP-KDO) complex in chain *A* and C5P complex in chain *B*, 1gqc; Jelakovic *et al.*, 1996; Jelakovic & Schulz, 2001, 2002], L-CKS from *E. coli* (apo, PDB code 1vh1; Badger *et al.*, 2005) and CKS from *Haemophilus influenzae* (apo, PDB code 1vic; CMP-KDO complex, 1vh3; Badger *et al.*, 2005) have been reported. Until now, however, no structure of any CKS bound with the substrate KDO alone has been reported. In order to provide the missing structural information, we have determined the crystal structure of CKS from *H. influenzae* complexed with KDO. It shows that the KDO bound to the active site exists as the β-pyranose form in the $^5C_2$ chair conformation. This structural information has significant implications for the catalytic mechanism.

2. Materials and methods

2.1. Protein expression and crystallization

We have previously reported the overexpression, crystallization and X-ray data collection of *H. influenzae* CKS (Ku *et al.*, 2003). The crystallographic asymmetric unit contains two monomers of *H. influenzae* CKS (Ku *et al.*, 2003).

2.2. Structure determination and refinement

Initially, we attempted to solve the structure by molecular replacement using the *E. coli* CKS structure as a probe, but the molecular-replacement solutions could not be refined to reasonably low R_{work} and R_{free} values. Therefore, we determined the structure using phase sets from both single-wavelength anomalous diffraction (mean figure of merit = 0.24 for 30–2.30 Å data) and molecular replacement. Eight of the ten expected Se atoms of two monomers (chains *A* and *B*) in each asymmetric unit were located with the program *SOLVE* (Terwilliger & Berendzen, 1999) and the phases were calculated with *RESOLVE* (Terwilliger, 2000). Model building was performed using the program *O* (Jones *et al.*, 1991). The model was refined with the program *CNS* (Brünger *et al.*, 1998) and several rounds of model building, simulated annealing, positional refinement and individual *B*-factor refinement were performed. Water molecules were added using the program *CNS*. The refined model revealed a large conformational difference in the C-terminal helical region (residues 230–254) of chain *A*, thus explaining our difficulty in refining the molecular-replacement solution.

3. Results and discussion

3.1. Model quality and structural comparisons

The refined model of the selenomethionine-substituted enzyme gave R_{work} and R_{free} values of 18.8% and 25.6%, respectively, for 30–2.30 Å data. It includes 492 residues in two monomers of *H. influenzae* CKS, two KDO molecules, one PEG 400 molecule and 162 water molecules in the crystallographic asymmetric unit (Table 1). All of the modelled nonglycine and nonproline residues are in the most favoured regions (90.43%), additional allowed regions (9.33%) or generously allowed regions (0.24%) of the Ramachandran plot. 16 residues (Ala11–Gly26) of chain *A* as well as the eight-residue tag (LEHHHHHH) at the C-terminus of the recombinant enzyme for both chains *A* and *B* were disordered and are missing from the model.

The two chemically identical subunits of the dimeric *H. influenzae* CKS adopt different conformations in the crystal. The root-mean-square (r.m.s.) deviation between chains *A* and *B* is 3.5 Å for 254 C^{α} atoms, with the C-terminal helical region (Val236–Asn254) displaying a very large r.m.s. deviation of 19.4 Å and a maximum deviation of 24.6 Å at Ala241. The C-terminal helical region of chain *A* is in a previously unobserved 'open' conformation, whereas that of chain *B* is in a 'closed' conformation as in all the reported CKS structures (Fig. 1*a*). When we exclude residues Val236–Asn254 from the comparison, the r.m.s. deviation is only 1.2 Å for 235 C^{α} atom pairs. This result demonstrates that the difference in the conformation of the C-terminal helical region between chains *A* and *B* has no major impact on the structure of the rest of the polypeptide chain. It is likely that the observed conformational variability of the C-terminal helical region has little

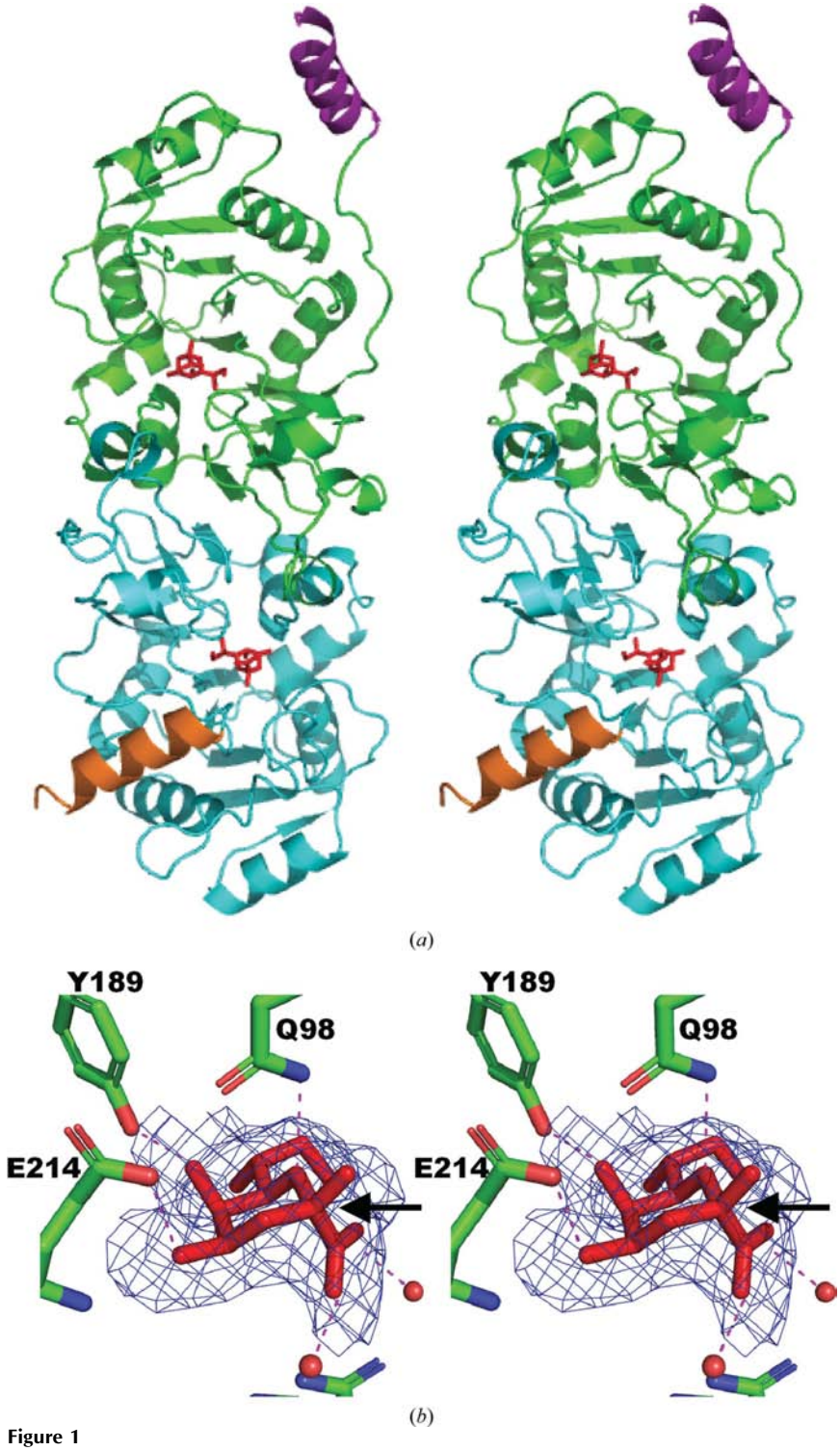

Figure 1
Structure of *H. influenzae* CKS bound with the substrate KDO. (*a*) Ribbon diagram of the dimer in stereo. Chains *A* and *B* are coloured green and cyan, respectively. The C-terminal α-helical region is highlighted in magenta and orange for chains *A* and *B*, respectively, to show the different conformations. These figures were drawn using the program *PyMOL* (http://www.pymol.org/). (*b*) Stereoview of KDO bound to the active site in chain *A*. The ($F_o - F_c$) OMIT electron-density map (coloured blue) contoured at 1.0σ is superimposed on the refined model. KDO (red) is shown as a stick model and the bound water molecules are shown as red balls. The magenta dotted lines indicate hydrogen bonds. The arrow indicates the anomeric centre at C2 of KDO.

Table 1
Refinement statistics.

Resolution range (Å)	30.0–2.30
No. of reflections (working/free set)	32140/3386
No. of residues	492
No. of water molecules	162
No. of KDO molecules	2
No. of PEG 400 molecules	1
R_{work}/R_{free}† (%)	18.8/25.6
R.m.s. deviations from ideal geometry	
Bond lengths (Å)	0.0058
Bond angles (°)	1.30
Average *B* factor (Å^2)	
Protein non-H atoms	28.4
Water molecules	30.4
KDO (in chain *A*/*B*)	21.1 (19.5/22.8)
PEG 400	45.4

† $R = \sum ||F_{obs}| - |F_{calc}|| / \sum |F_{obs}|$, where R_{free} is calculated for a randomly chosen 10% of reflections which were not used for structure refinement and R_{work} is calculated for the remaining reflections.

biological relevance. Chain *A* of our KDO-bound model shows an r.m.s. deviation of 3.0–3.1 Å for 237 C^{α} atoms in the apo structure of *H. influenzae* CKS (PDB code 1vic; Badger *et al.*, 2005). Chain *B* of our KDO-bound structure shows an r.m.s. deviation of 1.9–2.1 Å for 237 C^{α} atoms in the apo structure of *H. influenzae* CKS (PDB code 1vic). The significantly larger difference for chain *A* of our model is again a consequence of the 'open' conformation of its C-terminal helical region.

3.2. Substrates bound in the active site exist as the β-pyranose form

In the active site of our *H. influenzae* CKS structure, both chains *A* and *B* are bound with the substrate KDO, which is well defined by the electron density (Fig. 1*b*). Both KDO molecules exist as the β-pyranose form in the $^5C_2$ chair conformation (Fig. 1*b*). The β-pyranose form of KDO is a minor form in solution (Kohlbrenner & Fesik, 1985). The O atoms of KDO make hydrogen bonds to the NE2 atom of Gln98, the NH2 atom of Arg157, the ND1 atom of His185, the terminal O atom of Tyr189, the main-chain N atom of Glu214 and the NE2 atom of Gln215 in both chains *A* and *B* (Fig. 1*b*). These KDO–protein interactions are highly similar to those in the CMP-KDO complex structure of *H. influenzae* CKS (PDB code 1vh3; Badger *et al.*, 2005).

^{13}C NMR spectroscopy demonstrated that the β-pyranose form of KDO is the preferred substrate and that KDO retains the β-configuration when linked in the product CMP-KDO (Kohlbrenner & Fesik, 1985). The latter conclusion was made under the assumption that KDO is in the $^5C_2$ ring conformation (Kohlbrenner & Fesik, 1985). Subsequently, the crystal structure of *H. influenzae* CKS (PDB code 1vh3; Badger *et al.*, 2005) confirmed that KDO in CMP-KDO is in the β-configuration. Results of ^{31}P and ^{13}C NMR studies were consistent with a nucleophilic displacement mechanism for CKS (Kohlbrenner *et al.*, 1987). The structural information on the substrate complex of *H. influenzae* CKS reported in this study provides a strong line of evidence that supports the proposed catalytic mechanism.

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