Biochemical and Biophysical Research Communications 452 (2014) 789–794

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Kdo hydroxylase is an inner core assembly enzyme in the Ko-containing lipopolysaccharide biosynthesis

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ARTICLE INFO

Article history:
Received 22 August 2014
Available online 6 September 2014

Keywords:
Ko formation
Kdo hydroxylase
LPS inner core assembly
Burkholderia LPS
$Fe^{2+}/O_2/\alpha$-KG dependent dioxygenase

ABSTRACT

The lipopolysaccharide (LPS) isolated from certain important Gram-negative pathogens including a human pathogen *Yersinia pestis* and opportunistic pathogens *Burkholderia mallei* and *Burkholderia pseudomallei* contains D-glycero-D-*talo*-oct-2-ulosonic acid (Ko), an isosteric analog of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). Kdo 3-hydroxylase (KdoO), a Fe^{2+}/α-KG/O_2 dependent dioxygenase from *Burkholderia ambifaria* and *Yersinia pestis* is responsible for Ko formation with Kdo_2-lipid A as a substrate, but in which stage KdoO functions during the LPS biosynthesis has not been established. Here we purify KdoO from *B. ambifaria* (BaKdoO) to homogeneity for the first time and characterize its substrates. BaKdoO utilizes Kdo_2-lipid IV_A or Kdo_2-lipid A as a substrate, but not Kdo-lipid IV_A *in vivo* as well as *in vitro* and Kdo-(Hep)kdo-lipid A *in vitro*. These data suggest that KdoO is an inner core assembly enzyme that functions after the Kdo-transferase KdtA but before the heptosyl-transferase WaaC enzyme during the Ko-containing LPS biosynthesis.

1. Introduction

The outer membrane of Gram-negative bacteria is an asymmetric lipid bilayer that consists of phospholipids in its inner leaflet and lipopolysaccharide (LPS) in its outer leaflet and functions as a permeability barrier against hydrophobic compounds, such as detergents, bile salts, and antibiotics [1]. LPS is composed of three major parts: the hydrophobic lipid A (endotoxin), a non-repeating core oligosaccharide including two 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residues, and an O-antigen polymer [2,3].

The *Burkholderia cepacia* complex (BCC) comprises 17 closely related species that are isolated from diverse ecological niches, such as soils, rhizospheres, water, plants, fungi, animals, and infected humans [4]. Some members of the BCC have been used for efficient plant growth promotion [5] and bioremediation [6]. On the other hand, other members of the BCC are opportunistic human pathogens that can cause severe necrotizing pneumonia and septicemia in cystic fibrosis patients and in immuno-compromised individuals [7]. These Gram-negative bacteria synthesize an unusual isosteric analog of Kdo, known as D-glycero-D-*talo*-oct-2-ulosonic acid (Ko) [8–12], in which the axial hydrogen atom at the 3-position is replaced with a hydroxyl group [8] (Fig. 1A). We previously reported that heterologous expression of Kdo 3-hydroxylase (KdoO) from *Burkholderia ambifaria* (*B. ambifaria*) and *Yersinia pestis* (*Y. pestis*) in heptosyl transferase-deficient *Escherichia coli* WBB06 elicits hydroxylation of the 3-deoxy carbon in the outer Kdo unit of Kdo_2-lipid A, resulting in Ko formation. KdoO exhibits

Abbreviations: α-KG, alpha-ketoglutarate; Ara4N, 4-amino-4-deoxy-L-arabinose; BaKdoO, *Burkholderia ambifaria* KdoO; BCA, bicinchoninic acid; BCC, *Burkholderia cepacia* complex; BSA, bovine serum albumin; DEAE-cellulose, Diethylaminoethyl cellulose; DTT, dithiothreitol; EcKdtA, *Escherichia coli* KdtA; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; FPLC, fast protein liquid chromatography; Hep, heptose; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HiKdtA, *Haemophilus influenza* KdtA; IPTG, isopropyl β-D-1-thiogalactopyranoside; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; KdtA, Kdo transferase; KdoO, Kdo hydroxylase; Ko, D-glycero-D-*talo*-oct-2-ulosonic acid; LPS, lipopolysaccharide; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TLC, thin layer chromatography; WaaC, heptosyl transferase to Kdo_2-lipid A; WaaF, heptosyl tansferase to Kdo-(Hep)Kdo-lipid A; YpKdoO, *Yersinia pestis* KdoO.

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http://dx.doi.org/10.1016/j.bbrc.2014.08.153
0006-291X/

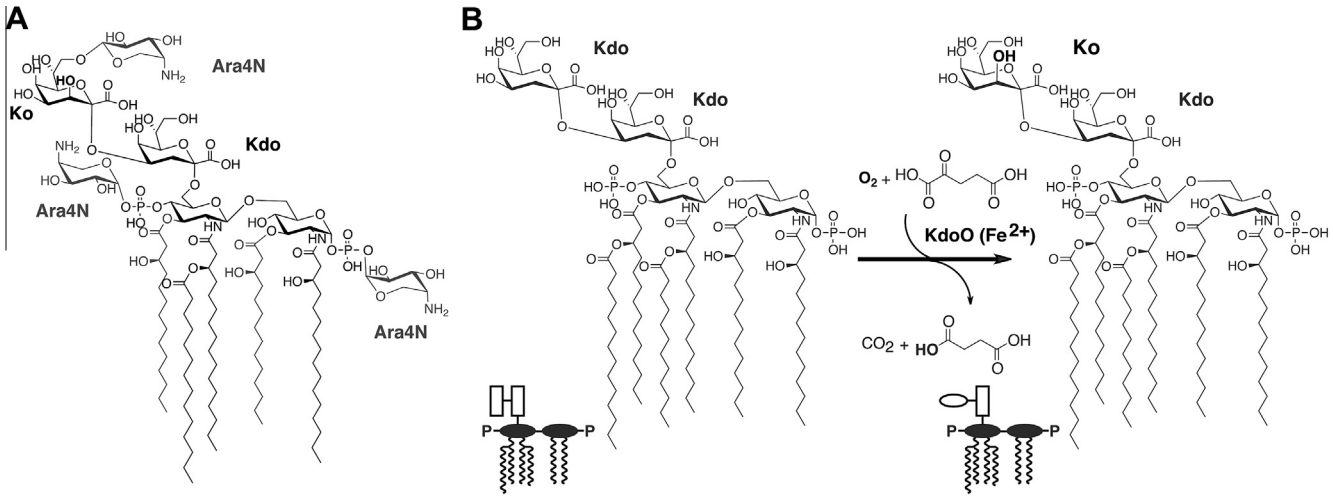

Fig. 1. (A) Ko-Kdo-lipid A structure of BCC. Gray colored residues are present in non-stoichiometric amounts. (B) Conversion of Kdo to Ko by KdoO. Kdo, *black boxes*; Ko, *empty oval*; glucosamine, *filled gray ovals*; acyl chains, *gray squiggles*: Phosphate group, *P*.

hydroxylase activity in an O_2, Fe^{2+} and α-ketoglutarate (α-KG) dependent manner [13] (Fig. 1B). KdoO belongs to the Domain of Unknown Function 2843 (DUF2843) family (Accession Number: PF11004, http://pfam.sanger.ac.uk/family/PF11004.3) that was annotated as the bacterial protein family with unknown function. KdoO homologues are found in human pathogens such as *Burkholderia mallei, Burkholderia pseudomallei, Klebsiella pneumoniae*, and *Coxiella burnetii*, in plant pathogens such as *Ralstonia solanacearum*, and in some environmental organisms such as *Methylobacterium extorquens*. [13]. It has been speculated that the extra OH group in Ko-containing LPS may provide an advantage under stressful environmental conditions by facilitating hydrogen bonding between adjacent LPS molecules in the outer membrane, and enhance or modulate the binging of LPS to TLR4/MD2 [13,14]. In addition, Ko-Kdo-lipid A was shown to be more resistant to mild acid hydrolysis than Kdo_2-lipid A *in vitro*, demonstrating the enhanced chemical stability of the Ko-Kdo and (Ko-Kdo)-lipid A linkages [13]. Therefore, understanding Ko formation in LPS assembly will provide insights into the biochemical and physiological role of outer membrane biogenesis. While KdoO belongs to the Fe^{2+}/α-KG/O_2 dependent dioxygenase family, it does not share high sequence similarity with any characterized Fe^{2+}/α-KG/O_2 dependent dioxygenase and has never been purified and characterized before.

Here, we report the purification of *B. ambifaria* KdoO (BaKdoO) to near homogeneity. After stabilizing its activity, we establish that KdoO is an inner core assembly enzyme and functions after the Kdo transferase but before the heptosyl transferase in the Ko-containing LPS biosynthesis.

2. Experimental: materials and methods

2.1. Materials

Chloroform, methanol, and silica gel 60 (0.25 mm) thin layer chromatograph (TLC) plates and high-performance analytical TLC (HPTLC) plates were from EMD Chemicals (Gibbstown, NJ). Tryptone, yeast extract, and agar were from BD Sciences (Franklin Lakes, NJ). Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was from Invitrogen (Carlsbad, CA). [γ-^{32}P]ATP (3 mCi/nmol) and γ-$^{32}P_i$ were from PerkinElmer (Waltham, MA). All other chemicals including α-KG, $Fe(NH_4)_2(SO_4)_2$, were of reagent grade. Purified Kdo_2-lipid A was obtained from Avanti Polar Lipids (Alabaster, AL).

2.2. Bacterial strains

Bacterial strains used in this study are described in supporting Table SI.

2.3. Molecular biology techniques

Protocols for handling DNA and preparing *E. coli* cells for electroporation were those of Sambrook and Russell [15]. Chemical transformation-competent *E. coli* cells were prepared according to the method of Inoue et al. [16].

2.4. Plasmid constructions and transformations into E. coli C41(DE3), CMR300, JW3596, or JW3595

A C-terminally His_6-tagged pBAKdoO-His_6 was constructed using primers prHSC167 (5′-GGCGCAGCATATGAGCGAATCCCAGAT CATCGA-3′) and prHSC171 (5′-GCAGAAGCTTAACCAGCGCCCGGC-3′), and the pET21b vector. The resulting plasmid was confirmed with primers T7F (5′-TAATACGACTCACTATAGGG-3′) and T7R (5′-GCTAGTTATTGCTCAGCGG-3′) and transformed into C41(DE3) [17]. pBaKdoO-HiKdtA was constructed with the extended PCR method described in reference [18] using primer pairs prHSC167/prHSC229 (5′-GCAAGCTGGTATAAAAAAAA CGCCACATTG GTATAT CTCCTTCTTATCAAACCAGCGCCCGG-3′, and prHSC228 (5′-CCGG GCGC TGGTTTGATAAGAAGGAGATATACCAATGTGGCGTTTTTTTTAT ACCAGCTTGC-3′)/prHSC7 (5′-GCAGAAGCTTTCATACATTGCGCTCCA AATAAGG TTTT-3′), with pBaKdoO.1 and pHiKdtA (Table S1) as PCR templates, respectively. The resulting PCR products from both reactions were mixed in a 1:1 ratio and used as a template for the second PCR, which was performed using primers prHSC167 and prHSC7. The resulting PCR products were ligated into the pBAD33.1. The sequence of the resulting plasmid was confirmed with primers 33F (5′-CTGTTTCTCCATACCCGTT-3′), 33R (5′-AATTCT GTTTTATCAGACCGCTT-3), and prHSC33 (5′-TGAGATCATATTTAATA TTGCCCGTGATATTCA-3′). These plasmids, pHiKdtA containing *Haemophilus influenza kdtA* [18] and pBAKdo-HiKdtA, were transformed into CMR300 [19], JW3596, and JW3595 [20].

2.5. Purification of BaKdoO-His_6

The overall purification scheme for BaKdoO-His_6 is shown in Fig. S2. C41(DE3)/pBaKdoO-His_6 was grown, induced at 18 °C for 20 h with 1 mM IPTG. The cells were then harvested and washed