Characterization of *Escherichia coli* D-arabinose 5-phosphate isomerase encoded by *kpsF*: implications for group 2 capsule biosynthesis

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In *Escherichia coli*, there are multiple paralogous copies of the enzyme API [A5P (D-arabinose 5-phosphate) isomerase], which catalyses the conversion of the pentose pathway intermediate Ru5P (D-ribulose 5-phosphate) into A5P. A5P is a precursor of Kdo (3-deoxy-D-*manno*-octulosonate), an integral carbohydrate component of various glycolipids coating the surface of the OM (outer membrane) of Gram-negative bacteria, including LPS (lipopolysaccharide) and many group 2 K-antigen capsules. The K-antigen-specific API KpsF has been cloned from the uropathogenic *E. coli* strain CFT073 and its biochemical properties characterized. Purified recombinant KpsF [K-API (K-antigen API)] is tetrameric and has optimal activity at pH 7.8.

The enzyme is specific for A5P and Ru5P, with K_m (app) values of 0.57 mM for A5P and 0.3 mM for Ru5P. The apparent k_{cat} in the A5P to Ru5P direction is 15 and 19 s^{-1} in the Ru5P to A5P direction. While most of the properties are quite similar to its LPS API counterpart KdsD, the catalytic constant is nearly 10-fold lower. K-API is now the second Kdo biosynthetic related gene that has been characterized from the *kps* group 2 capsule cluster.

Key words: D-arabinose 5-phosphate isomerase (API), 3-deoxy-D-*manno*-octulosonate (Kdo), *Escherichia coli*, group 2 capsule biosynthesis, K-antigen, lipopolysaccharide.

INTRODUCTION

The surface of the OM (outer membrane) in Gram-negative bacteria is the main interface through which the bacterium senses and responds to its environment. The surface of the OM of *Escherichia coli* is decorated with a number of diverse polysaccharides, including LPS (lipopolysaccharide), enterobacterial common antigen and various capsular polysaccharides. In *E. coli* alone, there are over 80 distinct capsular polysaccharides collectively known as K-antigens [1]. Capsular polysaccharides are the outermost cell-surface structure, and accordingly they have been implicated as key components in many of the initial host–pathogen interactions [2]. K-antigens have been postulated to protect the bacterium both directly by conferring resistance to non-specific host defence mechanisms (complement cascade) and indirectly by masking the presence of underlying molecules, which are more immunostimulatory, such as LPS (endotoxin), from triggering an immune response.

K-antigens are classified into four groups according to various genetic and biosynthetic criteria [3]. Most of the pathogenic extraintestinal isolates associated with infection belong to group 2. Group 2 capsules are encoded by the *kps* gene cluster located on a pathogenicity island near *serA*, and share a common genetic organization that is subdivided into three regions [4]. Region 1 consists of six co-transcribed genes (*kpsFEDUCS*), while region 3 contains two genes (*kpsMT*) convergently transcribed with respect to region 1. Region 3 contains the ATP-binding-cassette transporter and, together with region 1 genes, is believed to be involved in the export of the nascent polymer from the cytoplasmic face of the inner membrane to the OM surface [5]. Region 2 is flanked by regions 1 and 3 and contains a variable number of serotype-specific genes encoding protein products responsible for the synthesis and assembly of the saccharide portion of the K-antigen repeat unit. Much work has been done

on the transcriptional regulation of the *kps* locus in the prototype group 2 capsule systems from *E. coli* K1 and K5 [6–9]. Expression is under the control of multiple global regulators, including IHF (integration host factor), the nucleoid-associated protein H-NS, BipA and the anti-terminator RfaH, resulting in the co-ordinated repression of capsule synthesis at temperatures below 20 °C and induction at 37 °C. Thermoregulation has clear implications with respect to virulence during infection.

While much is known about the gene functions of the *kps* cluster and of the regulatory network controlling their expression, details concerning the mechanism of ligation of the K-antigen polysaccharide to the lipid anchor acceptor remain obscure [3]. Schmidt and Jann [10] originally reported the identity of the lipid anchor as a single Kdo (3-deoxy-D-*manno*-octulosonate) residue attached to a 1,2-dipalmitoylphosphatidic acid, suggesting that Kdo may serve a universal role in group 2 capsule biosynthesis by linking the polysaccharide region to the lipid domain, reminiscent of Kdo's well-studied essential role in LPS biosynthesis [11]. Accordingly, there is an increase in specific activity levels of CMP-Kdo synthetase (KpsU) in extracts from group 2 capsule strains only when grown at permissive temperatures [12]. In addition to KpsU, a second potential Kdo biosynthetic gene from the *kps* cluster (*kpsF*) was initially identified based on its ability to restore LPS biosynthesis in a *Neisseria meningitidis kdsD* construct [13], which was then successfully repeated in a *kdsD* mutant of *Yersinia pestis* [14]. KdsD (formerly YrbH) is an API [A5P (D-arabinose 5-phosphate) isomerase] from the LPS pathway that catalyses the 1,2-aldo/keto isomerization of Ru5P (D-ribulose 5-phosphate) to A5P, a precursor of Kdo [15]. Despite the conservation of both KpsU and KpsF among all group 2 isolates, *E. coli* K1 (and K92) strains do not have Kdo substituted at the reducing end of their polysaccharides [3]. In order to begin to understand better the role of Kdo in capsule biogenesis, *kpsF* from the group 2 capsule strain *E. coli* CFT073 (O6:K2:H1)

[16] was cloned, and the biochemical properties of recombinant capsular K-API (K-antigen API) are reported for the first time. The properties of K-API differ in comparison with its LPS (KdsD, L-API) counterpart, suggesting that K-API may have additional or alternative roles besides producing A5P for Kdo synthesis.

EXPERIMENTAL

Materials

E. coli strain CFT073 (O6:K2:H1) was obtained from Professor Harry L. T. Mobley (University of Michigan). PCR primers were synthesized by Invitrogen. Thermal cycling was performed using an MJ Research PTC-200 Peltier thermal cycler. Failsafe™ PCR PreMix Selection kit was purchased from Epicentre (Madison, MI, U.S.A.). The Promega Wizard DNA purification kit was utilized for plasmid purification. Chemically competent *E. coli* XL1-Blue (Stratagene) or *E. coli* BL21(DE3) (Novagen) cells were used for plasmid transformations. Restriction enzymes and T_4 DNA ligase were purchased from New England Biolabs. DNA sequencing was performed by the University of Michigan Biomedical Resources Core Facility. Hi Load™ Q-Sepharose (16/10) fast flow for anion-exchange chromatography, Fast Desalting (HR 10/10) columns for metal analysis studies and Superose 12 (HR 10/30) columns for gel-filtration native protein molecular mass analysis were all obtained from Amersham Biosciences. The gel-filtration protein molecular mass marker kit (12–200 kDa) was from Sigma.

Cloning of *E. coli* CFT073 *kpsF*

The *kpsF* gene was directly amplified from the *E. coli* CFT073 genome using standard whole-cell PCR methodologies using the following forward (GGTGCTAGAATTCATATGTCTGAAAGA-CATTTACC) and reverse (GAATTCGGATCCAAGTTAGTCG-AAAATGCGCACAAGGCC) primers in Failsafe™ PCR 2X premix buffer 'G' (Epicentre). Primers were designed to incorporate NdeI and BamHI sites respectively and used to ligate the PCR product in the similarly restricted expression vector pT7-7 that had been pretreated with calf intestinal alkaline phosphatase. The ligation mixture was used to transform chemically competent *E. coli* XL1-Blue cells, and a transformant harbouring the correct pT7-*kpsF* plasmid was identified by restriction analysis and confirmed by DNA sequencing. The plasmid was transformed into *E. coli* BL21(DE3) cells for protein expression.

Overexpression and purification of recombinant KpsF

E. coli BL21(DE3) cells harbouring the pT7-*kpsF* plasmid were grown in 1 litre of 2YT medium [1.6 % (w/v) tryptone, 1 % yeast extract and 1 % NaCl] containing ampicillin (100 μg/ml) at 37 °C with shaking (250 rev./min). Once the culture reached the mid-exponential growth phase ($D_{600} \sim 0.7$–0.9), the culture was allowed to cool to 18 °C before being induced with isopropyl β-D-thiogalactoside at a final concentration of 0.4 mM. After 16 h of growth at 18 °C, the cells were harvested by centrifugation (6500 g, 15 min, 4 °C). The cell pellet (11.2 g wet weight) was suspended in 35 ml of buffer A (20 mM Tris/HCl and 1 mM dithiothreitol; pH 8.0) and then sonicated on ice (5 × 30 s pulses with 2 min pauses in between). Cellular debris was removed by centrifugation (40 000 g, 30 min, 4 °C) and the supernatant was decanted (40 ml; 640 mg of protein). The solution was loaded on to a Hi Load™ (16/10) Q-Sepharose fast flow column that had been pre-equilibrated with buffer A. The protein was eluted using a 0–600 mM gradient of NaCl in buffer A over

120 min. Fractions containing primarily KpsF (~ 35 kDa), as judged by SDS/PAGE, were pooled (24 ml; 315 mg of protein) and precipitated by the slow addition of an ammonium sulphate-saturated buffer A solution with stirring at room temperature (22 °C) until 35 % saturation was reached. The solution was clarified by centrifugation (40 000 g, 45 min, 22 °C), and the protein pellet was resuspended in buffer A. The solution was dialysed against two changes of 2 litres of buffer A for 24 h each at 4 °C. The preparation was greater than ~ 95 % homogeneous as judged by SDS/PAGE analysis. The final total yield of recombinant KpsF protein was 230 mg/l of cell culture. Purified enzyme was divided into aliquots and stored at −80 °C.

Assay of API activity

A 96-well microplate assay using the cysteine-carbazole colorimetric assay was used for all assays as previously described [15]. Briefly, a solution containing 25 μl of enzyme at various concentrations in buffer (100 mM Tris/HCl; pH 7.8) was incubated at 37 °C for 3 min in a 96-well reaction PCR plate using a Peltier thermal cycler. The reaction was initiated with 25 μl of an A5P solution and subsequently quenched after various time intervals with 50 μl of 12.5 M H_2SO_4. One unit of enzyme activity is defined as the conversion of 1 μmol of sugar phosphate per minute at 37 °C.

Molecular mass of recombinant KpsF

Protein samples were prepared by buffer exchange via overnight dialysis against 2 litres of 5 mM Hepes (pH 8.0) at 4 °C. The subunit mass of KpsF was determined by MALDI (matrix-assisted laser-desorption ionization)-MS on a VESTEC-2000 instrument using a sinipinic acid matrix by the University of Michigan Protein Structure Facility. The native molecular mass was determined by gel filtration utilizing a Superose 12 column (HR 10/30) with 20 mM Tris/HCl buffer (pH 8.0, 150 mM NaCl) as eluent. The elution volume was determined in triplicate for all samples and protein standards (12–200 kDa).

Metal content analysis

Enzyme samples as isolated above were prepared for metal analysis by extensive dialysis (48 h) against 2 litres of metal-free buffer (20 mM Trizma/HCl; pH 7.5) at 4 °C. EDTA-treated protein samples were prepared by first incubating enzyme in the presence of 10 mM EDTA for 2 h at 4 °C, and were subsequently desalted using a Fast Desalting column (HR 10/10) with 20 mM Tris/HCl (pH 7.5) as eluent. Bivalent metal content of the untreated or EDTA-treated samples was determined by high-resolution inductively coupled plasma-MS on a Finnigan MAT ELEMENT instrument at the Department of Geology, University of Michigan.

Effect of metals and pH on the activity of KpsF

Samples of KpsF as isolated and described above were diluted in 100 mM Tris/HCl buffer (pH 7.8) and incubated with various bivalent metals or EDTA at 20 μM for 30 min at 4 °C. Activity was assayed at 37 °C under saturating substrate conditions in triplicate with a 3 min reaction time (50 mM Tris/HCl, 80 nM KpsF, 10 mM A5P and 10 μM bivalent metal or EDTA).

The activity of KpsF in BTP (Bis-Tris propane) buffer (pH 6–9.75 in 0.25 pH unit intervals) at 37 °C was measured using the standard cysteine-carbazole colorimetric assay conditions. Activity was measured in triplicate with a 3 min reaction time (100 mM BTP, 80 nM KpsF, 1 mM EDTA and 10 mM A5P).

Substrate specificity

The substrate specificity of KpsF was tested with a panel of naturally abundant carbohydrates (50 mM Tris/HCl, pH 7.8, 1 mM EDTA, 90 nM KpsF and 10 mM substrate). After 10 min at 37 °C, reaction mixtures containing the potential alternative substrates D-arabinose, D-ribose 5-phosphate, D-glucose 6-phosphate, D-glucose 1-phosphate, D-glucosamine 6-phosphate or D-mannose 6-phosphate were quenched, and the presence of ketose was determined as outlined above using the colorimetric cysteine-carbazole assay, which detects both pentuloses and hexuloses [17]. Each carbohydrate was assayed in triplicate, along with appropriate controls that did not contain enzyme. The lower limit of detection for this assay is estimated to be less than 1 % of ketose formation when using an initial 10 mM substrate concentration.

Determination of kinetic parameters

Reactions were performed at 37 °C using the end-point microplate assay with substrate concentrations typically ranging from 0.2 K_m to 10 K_m. After 2 min, reactions (50 mM Tris/HCl, pH 7.8, 1 mM EDTA and 90 nM KpsF) were quenched, at which point approx. < 10 % of substrate had been consumed. Initial rates (v_0) were determined in triplicate and fitted to the standard Michaelis–Menten equation using non-linear least-squares regression to determine values for K_m and k_{cat}.

The equilibrium constant was determined using ^{31}P NMR as previously described [15]. Solutions containing either 5 mM A5P or 5 mM Ru5P in 50 mM Tris/HCl (pH 7.5) with 1 mM EDTA and 10 % (v/v) ^2H_2O were incubated in the presence of 900 nM KpsF at 37 °C. Reactions were periodically monitored by ^{31}P NMR using a Bruker Avance DRX-300 instrument with WALTZ16 proton decoupling. A 10 s delay (> 3 times the $T1_{A5P/Ru5P}$) was used during the acquisition to ensure complete relaxation of the phosphorus nucleus, allowing for direct integration and comparison of peak areas for the two different sugar phosphates under observation. Chemical shifts were referenced to an external phosphoric acid standard (0 p.p.m.). Once there was no change in peak ratios for both samples, spectra were acquired (64 scans). K_{eq} is reported for API in the direction of Ru5P product formation from A5P substrate ([Ru5P]/[A5P]).

Miscellaneous methods

Protein concentrations were determined using the Bio-Rad Protein Assay Reagent assay with BSA serving as the standard. One-dimensional SDS/PAGE was performed under reducing conditions with a 12 % gel using a Mini-PROTEAN II electrophoresis unit (Bio-Rad).

RESULTS

Cloning, overexpression and purification of recombinant KpsF

The protein sequence deposited at the NCBI database for KpsF from *E. coli* CFT073 is 339 amino acids. The gene was initially cloned according to the deposited reading frame with the alternative start codon GTG, but MALDI-MS analysis revealed two recombinant protein peaks (*m/z* 35 323 and 36 579; results not shown) corresponding to a difference of 1256 Da. The first 12 N-terminal amino acids upstream of an internal methionine have a similar mass (1252 Da), suggesting that the methionine was actually the correct start codon. Further, there is a canonical ribosomal binding sequence [18] (AAGGAG) optimally spaced 5 base-pairs upstream of the internal methionine. Accordingly, the *kpsF* gene was re-cloned from the internal methionine in order to encode a 327-amino-acid open reading frame.
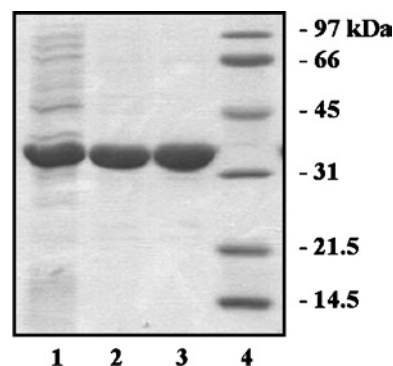
Figure 1   Purification of recombinant KpsF

Approximately 10 μg of protein was loaded on to an SDS/12 % polyacrylamide gel from each step of the purification. Lane 1, crude extract after sonication; lane 2, pooled fractions after Q-Sepharose anion-exchange chromatography; lane 3, post ammonium sulphate precipitation and dialysis; lane 4, molecular mass standards.

Recombinant KpsF was highly overexpressed using the pT7-7 expression vector. The vast majority of protein was localized in the cytoplasm, though a small fraction remained associated with the membrane fraction. Efficient purification to homogeneity was achieved in two steps using anion-exchange chromatography followed by ammonium sulphate precipitation (Figure 1). KpsF migrated as single band when analysed by SDS/PAGE under reducing conditions at approx. 35 kDa, and was estimated to be 95 % homogeneous. The specific API activity after dialysis was 25 units/mg.

Molecular mass determinations

MALDI-MS indicated the presence of a single protein species (M^{+1} 35 447 and M^{+2} 17 767), agreeing well with the calculated mass (35 486 Da), and suggesting that the internal methionine is indeed the correct native N-terminus. The native mass as determined by gel filtration was 145 ± 7 kDa, indicating that the quaternary structure of KpsF is tetrameric (~ 4.1 subunits).

Effect of metals and pH on the activity of KpsF

The metal content of KpsF as isolated was 0.85 ± 0.1 mol of bivalent metal cation per subunit, as determined by high-resolution inductively coupled plasma-MS, approx. 95 % of which was Ca^{2+} and Zn^{2+} ions. Samples that had been treated with EDTA and subsequently isolated by gel filtration contained only trace amounts of metals, including both Ca^{2+} and Zn^{2+} (< 0.01 mol per monomer), indicating that EDTA effectively chelates KpsF-bound metal cations.

As KpsF co-purified with nearly a stoichiometric amount of metal ions, the effect of bivalent metals on activity was investigated. The addition of EDTA to KpsF as isolated to remove the stoichiometric amount of bound cations, without removal of either the EDTA–metal complex or excess free EDTA, increased the observed activity approx. 2-fold (Figure 2A). The inclusion of surplus amounts of the late-transition metals (10 μM Zn^{2+}, Cd^{2+} and Hg^{2+}) significantly inhibited the activity of KpsF, though this inhibition could be reversed *in situ* by titration of the KpsF-bound metal with excess EDTA. In contrast, addition of certain other metals (Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Fe^{2+} and Co^{2+}) either had no appreciable effect on activity or was slightly stimulating. In order to maintain KpsF metal-free under the variety of assay conditions used to determine the optimum pH, substrate specificity and kinetic parameters, 1 mM EDTA was included in all reactions to chelate any bivalent metals.
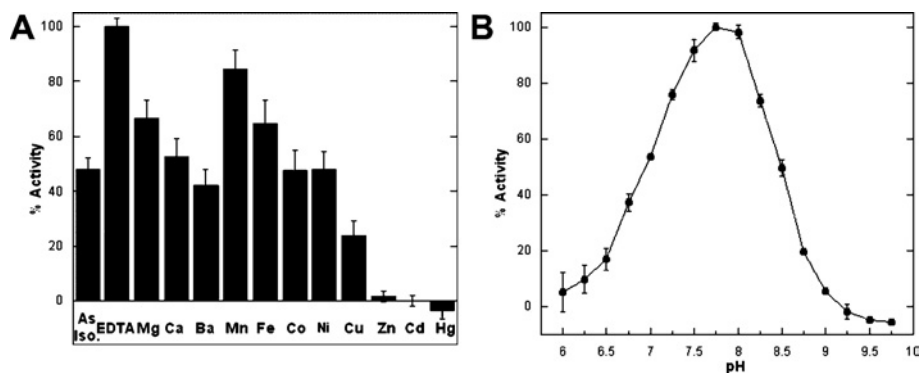
Figure 2   Effects of metals and pH on the activity of recombinant KpsF

(**A**) Metal analysis of recombinant KpsF. Activity of KpsF as isolated was measured at 37 °C in the presence of various metals or EDTA (50 mM Tris/HCl, pH 7.8, 80 nM KpsF, 10 mM A5P and 10 μM metal or EDTA). 'As Iso', as isolated. (**B**) The pH versus rate profile of recombinant KpsF (100 mM BTP, 80 nM KpsF, 1 mM EDTA and 10 mM A5P). All pH values were measured at 37 °C.

Table 1   Substrate specificity of recombinant *E. coli* KpsF

Substrates were assayed at 10 mM with 1 mM EDTA in 50 mM Tris/HCl (pH 7.8) for 10 min with 90 nM KpsF at 37 °C.

Substrate	Absorbance ratio*
A5P	2.5 ± 0.1
D-Ribose 5-phosphate	< 1
D-Arabinose	0.96 ± 0.06
D-Glucose 6-phosphate	1.01 ± 0.05
D-Mannose 6-phosphate	0.94 ± 0.06
α-D-Glucose 1-phosphate	1.00 ± 0.09
D-Glucosamine 6-phosphate	1.01 ± 0.01

* Ratio of absorbance at 540 nm of the sample to that of the control.

A plot of apparent maximum velocity versus pH produced a bell-shaped activity profile (Figure 2B), consistent with a diprotic mechanism involving both an acidic and a basic active site residue. The pH optimum was narrow and determined to be close to physiological pH at 7.75.

Substrate specificity

A panel of naturally abundant phosphorylated pentoses and hexoses were tested as potential substrates for KpsF (Table 1). However, D-ribose 5-phosphate, D-glucose 6-phosphate, D-glucose 1-phosphate, D-glucosamine 6-phosphate and D-mannose 6-phosphate were not converted into their respective ketoses as monitored by an increase in the ratio of absorbance at 540 nm of the sample to that of the control. D-Arabinose was also not a substrate, indicating that there is a requirement for phosphorylation. Within the limits of detection of the assay, KpsF thus appears to be a specific phospho-sugar aldo/keto isomerase catalysing the interconversion of A5P and Ru5P.

Kinetic parameters

Standard Michaelis–Menten kinetic analysis was applied to KpsF in order to determine the catalytic constants in both the forward (A5P to Ru5P) and the reverse (Ru5P to A5P) directions. The K_m was 0.57 ± 0.04 mM for A5P and 0.30 ± 0.03 mM for Ru5P, while the k_{cat} in the A5P to Ru5P direction was determined to be 15 ± 1 and 19 ± 2 s^{-1} in the Ru5P to A5P direction. The equilibrium constant was measured by integration of the ^{31}P NMR peaks (results not shown). The equilibrium lies well in favour of A5P, with the K_{eq} being equal to 0.48 ± 0.02 ([Ru5P]/[A5P]). The measured K_{eq}

constant was in agreement, within error, with the calculated K_{eq} using the kinetic constants as defined by the Haldane equation for reversible reactions ($K^{calc}_{eq} = 0.42$).

DISCUSSION

The role of KpsF, and Kdo in general, within group 2 capsule biogenesis has not been fully elucidated. In order to begin to understand better the function of Kdo, the *kpsF* gene from the uropathogenic strain *E. coli* CFT073 (O6:K2:H1) was cloned and characterized. KpsF was highly overexpressed as a soluble cytoplasmic protein (Figure 1). Previous attempts to overexpress the now identified KpsF reading frame cloned from *E. coli* K1 resulted in the nearly complete partitioning of recombinant protein into the membrane pellet, and thus required urea solubilization for purification [19]. In contrast, the vast majority of recombinant KpsF was readily soluble using the expression conditions reported here. KpsF certainly may be membrane-associated as was suggested [19], either through non-specific hydrophobic interactions with the membrane surface or perhaps through specific protein–protein interactions within a complex involving other integral membrane Kps proteins. As no other *kps* genes were co-overexpressed, KpsF may remain soluble without the presence of these Kps proteins to recruit it to the membrane, or simply once all the non-specific membrane association sites are filled, KpsF remains cytoplasmic. The difference may also stem from the fact that we did not use a cloning vector construct with a His tag because of the marked propensity of bivalent cations to exacerbate aggregation of KpsF.

Recombinant KpsF consistently co-purified with substantial quantities of bivalent cations, which could be removed *in situ* with excess EDTA chelating reagent. As the enzymatic activity actually increased upon sequestration of these cations, there does not appear to be a metal cofactor requirement for KpsF. The observed activity of the untreated enzyme preparation did slightly increase with certain other metals, particularly Mn^{2+}, though we attribute this to displacement of more inhibitory Zn^{2+} cations that had fortuitously bound during purification. A protein-stabilizing, auxiliary, or perhaps even regulatory role for the metal-binding site cannot be ruled out at this point, however. Reversible zinc inhibition at low micromolar concentrations has been observed in many other enzymes, and it has been proposed that metal ion inhibition may represent an under-appreciated post-transcriptional control mechanism *in vivo* [20]. It is unclear if the observed zinc inhibition in KpsF is biologically relevant, particularly

Table 2   Biochemical properties of API paralogues from *E. coli*

Property	*E. coli* API paralogue		
	L-API*	G-API†	K-API
K_m (A5P)	0.61 ± 0.06 mM	1.2 ± 0.1 mM	0.57 ± 0.04 mM
K_m (Ru5P)	0.35 ± 0.08 mM	0.64 ± 0.08 mM	0.30 ± 0.03 mM
k_{cat} (A5P to Ru5P)	157 ± 4 s^{-1}	218 ± 4 s^{-1}	15 ± 1 s^{-1}
k_{cat} (Ru5P to A5P)	255 ± 16 s^{-1}	242 ± 11 s^{-1}	19 ± 2 s^{-1}
K_{eq} (calc.)‡	0.47 (0.35)	0.47 (0.48)	0.48 (0.42)
Optimum pH	8.4	8.25	7.75
Specific for A5P/Ru5P§	Yes	Yes	Yes
Zn^{2+}/subunit‖	1.0 ± 0.1 mol	1.4 ± 0.2 mol	0.85 ± 0.1 mol
Inhibition by 10 μM Zn^{2+}¶	Yes	Yes	Yes
Activation by 10 μM EDTA	Yes	Yes	Yes
Subunit mass** (calc.)	35 104 Da (35 196 Da)	33 909 Da (34 031 Da)	35 447 Da (35 487 Da)
Native mass††	122 ± 5 kDa (tetramer)	133 ± 4 kDa (tetramer)	145 ± 7 kDa (tetramer)

* Data from [15].
† Data from [21].
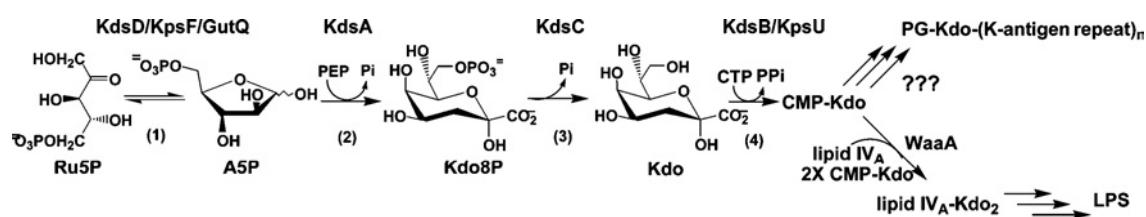‡ Measured by ^{31}P NMR (calculated from Haldane relationship [Ru5P]/[A5P]).
§ See the Experimental section for tested substrates.
‖ Amount of Zn^{2+} per monomer as determined by high-resolution inductively coupled plasma-MS.
¶ Less than 5 % activity remaining.
** Determined by electrospray ionization or MALDI-MS; calculated from protein sequence.
†† Determined by gel filtration.

Scheme 1   The Kdo biosynthetic pathway

Enzymes involved are (1) API (KdsD, KpsF and GutQ), (2) Kdo8P synthase (KdsA), (3) Kdo8P phosphatase (KdsC) and (4) CMP-Kdo synthetase (KdsB and KpsU). The LPS branch is initiated with the transfer of CMP-Kdo to lipid IV$_A$ acceptor by Kdo transferase (WaaA). Enzymes responsible for incorporating activated CMP-Kdo into the capsule are unknown. PG, phosphatidylglycerol.

when the cytoplasmic concentration of free unsequestered zinc in *E. coli* has been estimated to be in the femtomolar range [21].

The biochemical properties of KpsF are consistent with its identification as an API, and would suggest no other catalytic function. KpsF displayed reasonable affinity for both A5P ($K_m = 0.57 \pm 0.04$ mM) and Ru5P ($K_m = 0.30 \pm 0.03$ mM), and was specific for only these two substrates among a panel of other phosphorylated monosaccharides (Table 1). *E. coli* strains expressing group 2 capsules thus have three distinct API genes; *kpsF* from the K-antigen cluster (K-API), in addition to the two conserved API genes present in all *E. coli* strains {*kdsD* from the LPS (L-API) biosynthetic pathway [15] and *gutQ* from D-glucitol phosphotransferase operon (G-API) [22]}. While all three API paralogues catalyse the same reaction, a survey of their biochemical properties indicates that L-API and G-API are more similar to each other than to K-API (Table 2). Of particular difference are the values for the catalytic constant, with k_{cat} in each direction being more than 10-fold lower for K-API. The measured k_{cat} values were consistently lower across multiple independent protein preparations, regardless of the purification method. Further, measured activities in the crude KpsF extracts immediately after cell disruption were significantly lower than with the other APIs, suggesting that the decrease in catalytic efficiency is indeed real and not an artifact caused by differences in protein stability or purity. All three API proteins share greater than 40 % sequence identity, and yet the k_{cat} for K-API is an order of magnitude lower. A similar decrease in catalytic efficiency was

noted for the only other Kdo pathway enzyme on the *kps* operon, KpsU (CMP Kdo synthetase) from *E. coli* K5 [23]. When KpsU was compared with its LPS biosynthetic counterpart (KdsB), K_m values were 10-fold higher and the V_{max} 100-fold lower.

Kdo is a carbohydrate constituent of numerous group 2 capsule serotypes [24]. A single Kdo molecule can be found as the reducing sugar linking the K-antigen polysaccharide to the lipid α-glycerophosphate membrane anchor, as a repeating component of the capsular polysaccharide, or curiously, not at all in certain strains (K1 and K92) [3]. Although Kdo is not in the exported K1 polysaccharide, it may still play a role as an intermediate carrier for the polysaccharide chain, which is then subsequently displaced during the ligation of the lipid, as was proposed for the related capsule system from *Neisseria* [13]. Despite the prominent role of Kdo in capsule biogenesis, only two Kdo-related genes (*kpsF* and *kpsU*) are located on the *kps* operon (Scheme 1). Neither Kdo synthetase [25] nor Kdo phosphatase [26] is duplicated. Genetic studies in *E. coli* K-12 K5 have shown that KpsF and KpsU are not essential for capsule synthesis [27,28]. Paradoxically, a $\Delta kpsF$ mutant in an *E. coli* K-12 K1 hybrid resulted in an intermediate phenotype, where there was a 10-fold decrease in capsule polymer being transported to the surface [19]. Failure to clearly observe the $\Delta kpsF/\Delta kpsU$ phenotypes was reasonably attributed to partial complementation by their homologues. Further, we would expect that a clear phenotype will only be manifested when the capsule biosynthetic demand for Kdo exceeds the capacity of the LPS pathway enzymes to

supply it. These apparent discrepancies may therefore stem from differences in the kinetics of polysaccharide assembly apparatus which are unique to each individual K-antigen, i.e. intracellular pools of Kdo in a $\Delta kpsF$ construct become limiting for one but not for the other. It would be interesting to construct these same mutants in *E. coli* serotype strains that have Kdo in the repeat unit, where 40–60 % of the capsular polysaccharide mass consists of Kdo [29]. One would expect that the availability of Kdo would become rate-limiting, and therefore be able to directly observe the $\Delta kpsF$ phenotype. It is possible that all group 2 capsules arose from a prototype group 2 strain with Kdo in the repeat unit. Thus post acquisition via horizontal transfer of region 2 genes that do not direct the synthesis of a Kdo-containing polysaccharide, the functions of KpsF and KpsU have become redundant.

Probing the role of Kdo in capsule biogenesis in *E. coli* is complicated by the presence of complementing genes for both KpsU and KpsF. KpsU was reported to be membrane-associated [23], and perhaps may be a component of a Kps protein complex. An argument could be made that KpsU is present in order to generate the activated sugar nucleotide CMP-Kdo 'on-site' within the immediate proximity of the Kps protein complex in order to accommodate the remarkably short half-life of CMP-Kdo [30]. Further, the decreased catalytic competency of KpsU may ensure that if Kdo pools ever do become limited, there is a hierarchal distribution of Kdo in favour of KdsB, and thus to the essential LPS biosynthesis pathway. The presence of K-API may reflect the need to supplement the basal level of A5P produced during the capsule production phase, suggesting that the total intracellular API level dictates the net flux through the Kdo pathway. The existence of an alternative specific role for K-API in protein–protein interactions, as either a stabilizing component or signalling mechanism, cannot be ignored. However, the high sequence identity over the entire length of the reading frame between the API paralogues would suggest that if such a role exists, they would be able to complement each other in this hypothetical function along with the established catalytic one. We recently constructed a viable ΔKdo strain in *E. coli* K-12 [31]. The construction of group 2 capsule hybrids in this host will hopefully enable further probing of the role of KpsF and Kdo in capsule biogenesis.

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