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Binding of the natural substrates and products to KDO8P synthase: ^{31}P and ^{13}C solution NMR characterization

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Abstract

Proton decoupled ^{31}P and ^{13}C solution NMR experiments were applied to mixtures of 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (KDO8P) synthase, with each of its natural substrates, phosphoenolpyruvate and arabinose-5-phosphate (ASP), and product KDO8P to identify the formation of the enzyme–substrate and enzyme–product complexes. Effects arising from ligand interactions with the enzyme are reported via chemical shifts and line broadening with respect to those of the free ligands in solution, depending on the strength and dynamics of binding under thermodynamic equilibrium conditions. The characterization was done both at low and high field spectrometers, 200 and 500 MHz (^{1}H frequencies), and in cases of ^{31}P NMR measurements, it was demonstrated that only the low field spectrometer is capable of providing direct experimental evidence on the enzyme–ligand interactions. Since both the substrate A5P and the product KDO8P exhibit multiple anomeric forms in solution, evidence for the preference of recognition and binding of particular forms is sought.

Keywords: KDO8P synthase; Biosynthesis of KDO; Lipopolysaccharides; NMR of enzyme–substrate complexes

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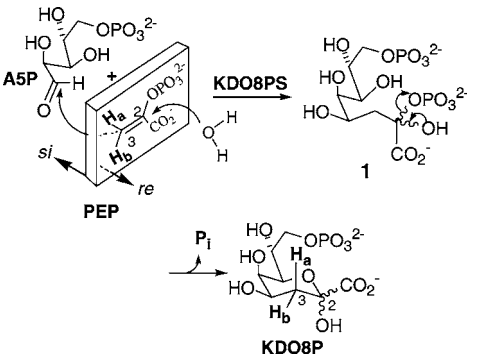
1. Introduction

The enzyme 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (KDO8P)[2] synthase (KDO8PS) (EC 4.1.2.16) catalyzes the condensation reaction between phosphoenolpyruvate (PEP) and D-arabinose-5-phosphate (A5P) to form KDO8P and inorganic phosphate [1] (Scheme 1). This important enzymatic reaction controls the carbon flow in the biosynthetic formation of an unusual 8-carbon sugar 3-deoxy-D-*manno*-2-octulosonate (KDO). KDO is an essential constituent of the lipopolysaccharide of all Gram-negative bacteria [2] and plays a crucial role in their assembly process [2,3]. Since KDO is a site-specific constituent found only in Gram-negative organisms and plants, and is required for lipid A maturation and cellular growth, the inhibition of its production sets an attractive target for the design of novel antibacterial drugs.

The catalytic mechanism of KDO8PS has been studied extensively. Although earlier studies [4,5] have established that the reaction proceeds with the unusual cleavage of the C–O bond of PEP, since the net reaction is essentially irreversible [4], the enzyme-bound intermediates cannot be examined under equilibrium conditions and, therefore, its elementary steps were not directly identified [6]. This fact has impeded the investigation of the mechanism of the synthase with its natural substrates, leading to the design and synthesis of various analogs of substrates and product, and their characterization with the enzyme [7–9]. Along with the cloning and overexpression of the *Escherichia coli* gene encoding for KDO8PS [10], these have greatly facilitated mechanistic studies [11].

Earlier studies, using stereospecifically 3-deuterio and 3-fluoro labeled analogs of PEP as alternative substrates of KDO8PS, have demonstrated that the condensation step is stereospecific, involving the attachment of the *si* face of PEP to the *re* face of the carbonyl of A5P [7,8]. By using 4-deoxy analog of A5P, 4-deoxy-A5P, which is structurally prohibited from undergoing ring closure, it was shown that this analog functions as an alternative substrate for the enzyme with a similar k_{cat} value to that of A5P and discriminates against compounds with furanose locked rings [9]. Thus, it was concluded that the substrate of KDO8PS must be the acyclic form of A5P. On the other hand, synthesis and characterization of A5P analogs lacking a hydroxyl group at positions 2 or 3 (2-deoxy- and 3-deoxy-A5P) were found to be non-reactive analogs, and exhibited no inhibition [12]. Since the latter analogs can undergo ring opening/closure, this observation alluded to the crucial importance of these hydroxyls (C2-OH and C3-OH) for the recognition and binding of A5P by the enzyme. More recent studies using rapid-quench techniques [6], including the synthesis and evaluation of the first acyclic bisubstrate inhibitor ($K_d = 0.4\,\mu M$) [13], supported the original hypothesis of Hedstrom and Abeles [4], suggesting that the reaction pathway proceeds through an acyclic bisphosphate intermediate **1** (Scheme 1).

[2] *Abbreviations used:* A5P, D-arabinose-5-phosphate; PEP, phosphoenolpyruvate; KDO8P, 3-deoxy-D-*manno*-2-octulosonate-8-phosphate; KDO8PS, 3-deoxy-D-*manno*-2-octulosonate-8-phosphate synthase; REDOR, rotational-echo double-resonance.



Scheme 1. Proposed mechanism for KDO8PS-catalyzed reaction.

In our earlier NMR work [14,15], we have reported the first direct identification of active site residues of KDO8PS with each of its natural substrates by solid-state REDOR NMR. The REDOR NMR data showed that both the α- and β-anomers of A5P, which constitute over 97% of its anomers in solution, are also the primary forms of A5P which are bound to the enzyme in the binary complex (KDO8PS–A5P). Since KDO8PS is capable of binding both the α- and β-anomers, it is conceivable that no enzyme involvement should be required for their distinction. Therefore, we concluded that the C1-linked hydroxyl group of A5P (α and β) has a minor role in the recognition and binding of A5P's cyclic forms, leaving this side of A5P free to undergo ring opening, which is essential for the progression of the catalytic reaction. In parallel, the first X-ray crystal structure of KDO8PS was determined in the presence of sulphate/phosphate ions replacing the natural substrates [16]. This study demonstrated that the enzyme is a 120-kDa homotetramer and each subunit has a fold of $(\beta/\alpha)_8$ barrel. Following this work, other structures of *E. coli* KDO8PS were solved [17,18], including the binary complexes [17] of the enzyme with the substrate PEP, and with the mechanism-based inhibitor, however to date, no crystallographic data were derived for the KDO8PS–A5P binary complex. The inhibition of the KDO8PS-catalyzed reaction by the product and several of its analogs was also examined [11,19]. The product, KDO8P, as well as its 2-deoxy-α- and β-pyranose analogs, were found to be weak inhibitors of the enzyme [11]. These analogs were found to bind to the enzyme competitively with respect to PEP, having K_i values of 470 and 303 μM, respectively. Based on the comparison of these data to the K_i value of the tautomeric mixture of the product KDO8P ($K_i = 590\,\mu M$), it was suggested that both the α- and β-pyranose anomers of KDO8P (65.8% and 3.1%, respectively, at neutral pH) [11] bind to the enzyme with a slight preference for the β-anomer, and that the C2 hydroxyl does not contribute to the binding.

As a part of our study on the structure–function relationship of KDO8PS, we present here a detailed account of solution NMR experiments, which were used to identify the formation of the binary complexes of the enzyme with the substrates, and product under thermodynamic equilibrium conditions. In this work ^{31}P and ^{13}C solution NMR characterization of the binary complexes of KDO8PS with each

of its natural substrates and with the natural products are described. This characterization is conducted at two field strengths, 200 and 500 MHz (^{1}H frequencies). Since both the substrate A5P and the product KDO8P exhibit multiple anomeric forms in solution, evidence for the preference of recognition and binding of particular forms is sought. This work complements our earlier solid-state NMR observation [15], as well as studies with synthetic analogs of the substrates and the product.

2. Materials and methods

2.1. General methods

A5P was prepared enzymatically according to the procedure of Whitesides [20]. [1-^{13}C]A5P was prepared from [1-^{13}C]D-arabinose (^{13}C, 96%, Cambridge Isotope Laboratories, CIL) and hexokinase (Sigma) by the same method as A5P. The potassium salt of PEP was prepared in large quantities as already described [21]. [2-^{13}C]PEP (^{13}C, 99%) was purchased from CIL. All other chemicals used in this study were purchased from Aldrich or from Sigma and were used without further purification.

2.2. Preparation of [2,4-$^{13}C_2$]KDO8P

The compound was prepared enzymatically from [2-^{13}C]PEP (2.4 mg, 11 μmol) and [1-^{13}C]A5P (7 mg, 23 μmol) as the substrates. In addition to the labeled substrates the enzymatic mixture contained 100 mM Tris–DCl buffer (prepared by dissolving solid Tris in D_2O and adjusting the pD with DCl to 7.3) and non-labeled PEP (1 mg, 5 μmol). The reaction was initiated by the addition of 1 U KDO8PS directly to the NMR tube containing the above mixture. The formation of the [2,4-$^{13}C_2$]KDO8P product was monitored by ^{31}P NMR and ^{13}C NMR. ^{13}C NMR (50.3 MHz, 100 mM Tris–HCl in D_2O, pD 7.3): δ_{C2}: 96.9 (α-pyr), 97.9 (β-pyr), 103.4 (β-fur), and 104.7 (α-fur); δ_{C4}: 66.8 (α-pyr), 68.0 (β-pyr), 71.5 (β-fur), and 72.8 (α-fur). ^{31}P NMR (81.0 MHz, 100 mM Tris–HCl in D_2O, pD 7.3): δ/ppm 4.69 (α-pyr), 4.87 (α-fur), and 5.01 (β-fur).

2.3. Overexpression and purification of KDO8PS

KDO8PS (specific catalytic activity 9 U/mg) was isolated from the overproducing *E. coli* DH5α (pJU1) strain, as previously described [9]. The recombinant enzyme was purified according to the method previously reported by Ray [1] with slight modifications [9]. All manipulations were carried out at 4 °C. All buffers used during the purification contained 0.1 mM dithiothreitol (DTT); the pH of the buffers was determined at 10 °C.

2.4. Enzyme activity assay

Unless otherwise stated, the enzyme activity was assayed in 1.0 mL of a reaction buffer consisting of 0.1 M Tris–HCl, pH 7.3, 0.2 mM PEP, and 0.5 mM A5P.

Following equilibration at 37 °C for 2 min, KDO8PS (10 μL, at a final concentration of approximately 30 nM) was added and the decrease in the absorbance difference between 232 and 350 nm (as internal reference) was monitored as a function of time (MS-DOS UV/VIS software). This method [22] is based on the absorbance difference at 232 nm between PEP ($\varepsilon = 2840\ M^{-1}\ cm^{-1}$) and the other substrates and products ($\varepsilon < 60\ M^{-1}\ cm^{-1}$) under the assay conditions. The initial rate was calculated from a linear least-squares fit to the first 30 s of the progress curve. One unit of the enzyme activity is defined as the amount that catalyzes the consumption of 1 μmol PEP/min at 37 °C. During the purification, the enzyme activity was monitored by the thiobarbituric acid assay method as previously reported [1,9]. Protein concentration was determined using Bio-Rad Protein Assay with bovine serum albumin as a standard.

Spectrophotometric measurements were made on a Hewlett-Packard 8452A diode array spectrophotometer, using 1-cm path-length cells with a thermostated cell holder and a circulating water bath at the desired temperature.

2.5. Preparation of apo-enzyme and enzyme complexes for NMR measurements

Purified KDO8PS (typically 70–80 mg protein) was exchanged into a buffer by extensive dialysis (24 h, with 4–6 buffer replacements), containing 100 mM Tris–HCl, pH 7.3, 0.1 mM DTT, and then followed by exchange of buffer to 100 mM Tris–HCl in D_2O, pD 7.3. The enzyme solution was concentrated by ultrafiltration through a centricon (10 kDa cut-off) to final concentrations of 4.6–5.3 mM in the same buffer. The enzyme concentration was determined according to the subunit molecular mass of 30 kDa (i.e., 1 mM enzyme is 30.0 mg/mL). KDO8PS complexes were prepared by direct addition of appropriate quantities of PEP, A5P, and KDO8P or their labeled analogs, [2-^{13}C]PEP and [1-^{13}C]A5P to the above enzyme solution in deuterated buffer.

2.6. NMR spectroscopy

Proton-decoupled ^{13}C NMR spectra were recorded on a Bruker AM-200 at 50.3 MHz, and on AM-500 spectrometer at 125.8 MHz, and the chemical shifts are reported (in ppm) relative to external standard of sodium 2,2-dimethyl-2-silapentane sulfonate ($\delta = 0.0$) in D_2O. Proton-decoupled ^{31}P NMR spectra with single-pulse excitation were recorded on a Bruker AM-200 at 81.0 MHz and AM-500 at 202.5 MHz. Chemical shifts are reported (in ppm) relative to external orthophosphoric acid ($\delta = 0.0$) in D_2O. All experiments were performed at 6 °C, using 5-mm high-resolution NMR tubes. All low field experiments were acquired with 2 s repetition time, except for measurements of KDO8PS–PEP binary complex for which 9 s repetition time was used. The high field experiments employed 2 s repetition time. Data acquisition time of the enzyme–ligand binary complexes between 3 and 7 h, and between 30 and 60 min, were employed at the low and high field spectrometers, respectively.

3. Results

In the following, identification of enzyme–substrate(s) and enzyme–product(s) interactions were sought by solution NMR. As will be shown, using proton-decoupled ^{31}P and ^{13}C NMR experiments, the effects induced by ligand interactions with the enzyme are reflected by either chemical shifts and/or line broadenings. At first, the study of enzyme–PEP binary complex is described, followed by that with A5P and with KDO8P.

3.1. KDO8PS–PEP binary complex

The 81.0 MHz ^{31}P NMR spectrum of PEP shown in Fig. 1a depicts −0.2 ppm peak. The ^{31}P NMR spectra of the binary complex of KDO8PS with PEP at 81.0 and 202.5 MHz spectrometers are shown in Fig. 1b and c, respectively. The low field spectrum (Fig. 1b) depicts a broadened peak (~100 Hz) at the same chemical shift as that of free PEP in solution, −0.2 ppm (Fig. 1a), while at the higher field (Fig. 1c) no peak is observed. The broadening observed at the low field is attributed to the binding of PEP to the enzyme via the dynamic equilibrium process $E + PEP \leftrightarrows E-PEP$, which at the high field broadens the peak beyond detection. This extensive broadening is attributed to the tight binding of PEP [9] ($K_m = 6\,\mu M$) at the most recessed part of the active site cavity [16,17]. It should be noted that the 100 Hz wide PEP-phosphate peak in spectrum 1b reflects an averaging of both exchanging forms, the bound and unbound forms of PEP, both possessing the same chemical shifts. Upon binding to the 120-kDa tetrameric protein [16], the bound species is broadened due to both the slowing of its motional characteristics as well as rendering them anisotropic, giving rise to non-vanishing residual interactions, e.g., shift anisotropy of the phosphate group [23].

In parallel, proton-decoupled ^{13}C NMR spectra were recorded. The 50.3 MHz ^{13}C spectrum of [2-^{13}C]PEP at is shown for a reference in Fig. 2a, depicting a peak

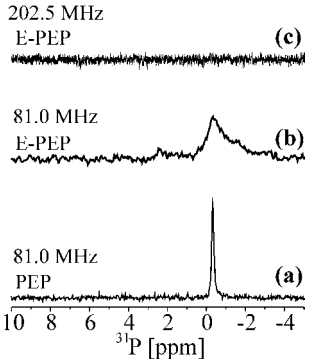


Fig. 1. ^{31}P proton-decoupled NMR spectra of: (a) PEP at 81.0 MHz, 64 scans (30 mM in 0.1 M Tris–D_2O buffer, pH 7.3); KDO8PS–PEP 1:1 binary complex (4.6 mM KDO8PS; and 4.6 mM PEP in 0.1 mM Tris–D_2O buffer, pH 7.3) at (b) 81.0 MHz, 5000 scans, and (c) 202.5 MHz, 512 scans.

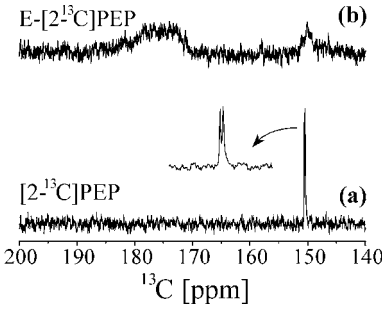


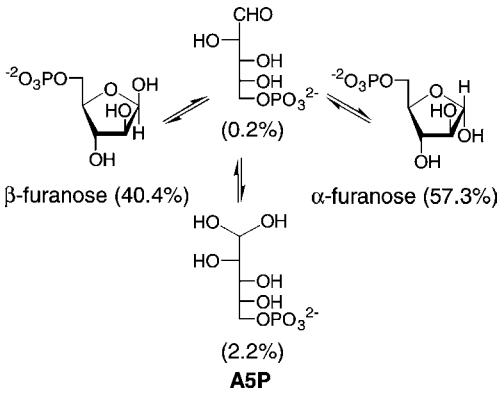
Fig. 2. ^{13}C proton-decoupled NMR spectra of: (a) [2-^{13}C]PEP at 50.3 MHz, 128 scans (30 mM in 0.1 mM Tris–D_2O, pH 7.3) and (b) KDO8PS–[2-^{13}C]PEP 1:1 binary complex at 125.8 MHz, 2024 scans (conditions as in Fig. 1b).

centered at 149.0 ppm (a doublet, $J_{PC} = 7.5$ Hz, inset). The KDO8PS–[2-^{13}C]PEP binary complex gave rise to similar spectra at the two fields, represented by the 125.8-MHz spectrum in Fig. 2b. In addition to the broad natural abundance ^{13}C enzyme carbonyls peak (170–185 ppm), the selected downfield spectral region shows the broadened resonance of the [2-^{13}C]PEP at 149.0 ppm, centered at about the same chemical shift as its free moiety. This broadening arises upon binding of PEP to the enzyme, as was discussed above for the ^{31}P spectra.

3.2. KDO8PS–A5P binary complex

Earlier studies [9], using 4-deoxy-A5P as an alternative substrate, have clearly demonstrated that KDO8PS acts upon the acyclic aldehyde form of its substrate A5P. It should be noted, however, that among the four different forms of A5P present in neutral solution, the aldehyde form is present at less than 0.3%, the α- and β-furanose anomers constitute 97.7%, and the acyclic hydrate form is present at ca. 2.0% (Scheme 2) [24]. Therefore, knowing whether the free aldehyde form or the open chain hydrate form of A5P is the only form being recognized and bound by the enzyme, or the enzyme can also recognize and bind the cyclic furanose forms of A5P, is important for the identification of the initial steps of the catalytic mechanism. For this purpose, in our recent work [15], applying solid state $^{13}C\{^{31}P\}$ REDOR NMR to the lyophilized KDO8PS–A5P binary complex, we found that the major forms of enzyme-bound A5P are A5P's cyclic forms.

In order to further substantiate this conclusion, and since the ^{31}P chemical shift of the phosphate moiety is not sensitive to the different anomeric forms of A5P (vide infra), we have subjected the binary complex of KDO8PS–[1-^{13}C]A5P to ^{13}C solution NMR characterization. Its proton-decoupled ^{13}C NMR spectrum (125.8 MHz) in Fig. 3b depicts two broadened peaks, representing the two cyclic α-furanose and β-furanose forms of A5P at 101.6 and 95.7 ppm, respectively. These chemical shifts are the same as those observed in Fig. 3a for free [1-^{13}C]A5P. No evidence for additional ^{13}C peaks, e.g., of the open forms of A5P (expected between 160 and



Scheme 2. Distribution of the different anomeric forms of A5P under neutral aqueous condition [24].

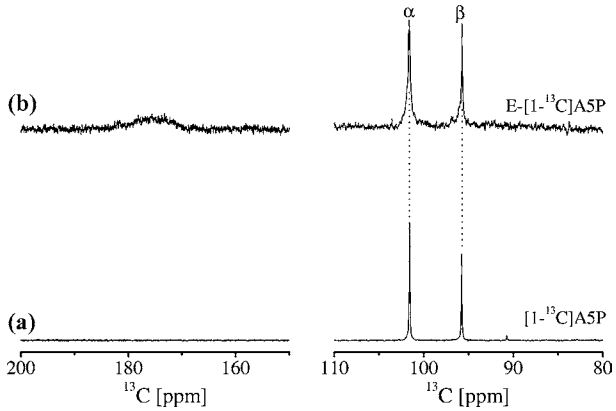


Fig. 3. ^{13}C proton-decoupled NMR spectra of: (a) [1-^{13}C]A5P at 50.3 MHz, 128 scans (10 mM in 0.1 mM Tris–D_2O, pH 7.3) and (b) KDO8PS–[1-^{13}C]A5P 1:1 binary complex at 125.8 MHz, 2024 scans (4.7 mM KDO8PS and 4.7 mM [1-^{13}C]A5P).

200 ppm), could be detected for the binary complex or for the free A5P. The absence of such peaks in the binary complex spectrum cannot support the notion that the open form would be the major bound form. The broadening of the α and β peaks in the binary complex relative to the free forms in solution, and the similar relative integrated intensities to those of free A5P, suggest that both the α- and β-furanose forms of A5P are being recognized and bound by the enzyme. This result is in accordance with our earlier solid-state NMR observations [15]. The reduced broadening observed for these two enzyme-bound forms of [1-^{13}C]A5P (Fig. 3b, ~100 Hz for α anomer) versus that observed for the enzyme-bound [2-^{13}C]PEP (Fig. 2b, ~250 Hz), are attributed to the smaller chemical shift anisotropy of [1-^{13}C]A5P (70 ppm, [25]) vs. [2-^{13}C]PEP (147 ppm [26]), and possibly also to A5P's weaker binding strength ($K_m = 26\,\mu M$) compared to that of PEP. Fig. 3b also shows somewhat

larger broadening of the α anomer peak vs. that of the β anomer. This observation reflects differences in the interactions of the two anomers with the synthase, whose nature needs further study.

In order to complete the characterization of the binary complex KDO8PS–A5P, ^{31}P solution NMR measurements were recorded at the 81.0 and 202.5 MHz NMR spectrometers. First, the reference proton-decoupled ^{31}P NMR of [1-^{13}C]A5P at 81.0 MHz is shown in Fig. 4a, depicting a 4.2 ppm peak of the phosphate group, and residual impurity peaks arising in the synthesis of A5P. Fig. 4b shows the ^{31}P NMR spectrum at 81.0 MHz of the binary complex KDO8PS–A5P obtained upon the addition of 0.8 equiv. A5P to the apo-enzyme, depicting a broad peak at 3.8 ppm and a sharper resonance at 2.2 ppm assigned to inorganic phosphate. The broad peak at 3.8 ppm is assigned to enzyme-bound A5P phosphate, upfield shifted compared to free A5P at 4.2 ppm (Fig. 4a). The substantial inorganic phosphate 2.2 ppm peak arises in this experiment from the cumulative action of KDO8PS as a phosphatase. Such residual phosphatase activity was also identified in the presence of PEP [27] (data not shown), yet to a lesser extent. As a consequence, the actual molar ratio, A5P/KDO8PS, is much lower than 0.8. This fact also explains the absence of a free A5P peak (at 4.2 ppm) in the spectrum of the KDO8PS–A5P binary complex (Fig. 4b), since at this reduced molar ratio most substrate is enzyme bound.

The ^{31}P solution NMR spectrum of KDO8PS–[1-^{13}C]A5P (1:1) binary complex at 202.5 MHz shown in Fig. 4c exhibits a major peak at 4.2 ppm, identified as free A5P-phosphate, and a minor peak at 4.5 ppm attributed to the impurity as was observed

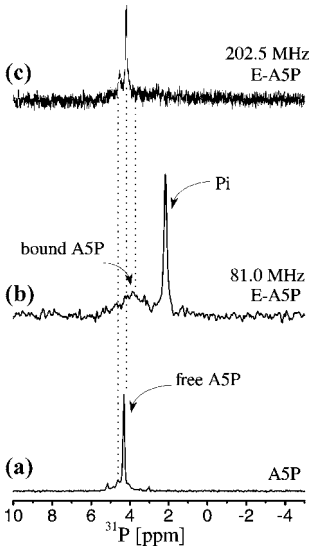


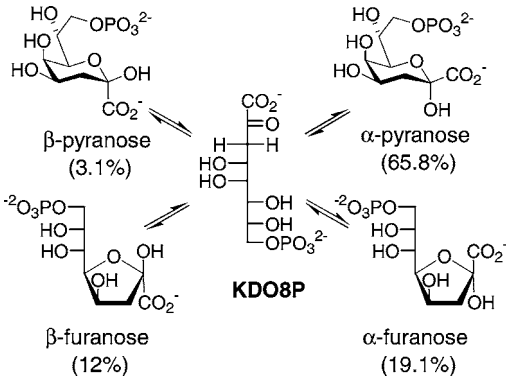
Fig. 4. ^{31}P proton-decoupled NMR spectra of: (a) A5P at 81.0 MHz, 64 scans (10 mM in 0.1 mM Tris–D_2O, pH 7.3). The minor peaks in the spectrum depict residual impurities arising in the synthesis of A5P; (b) KDO8PS–A5P 1:0.8 binary complex at 81.0 MHz, 4000 scans; and (c) KDO8PS–[1-^{13}C]A5P (1:1) binary complex at 202.5 MHz, 2024 scans (conditions of b and c as in Fig. 3b).

in the A5P reference spectrum (Fig. 4a). The presence of the 4.2-ppm free A5P phosphate peak at 202.5 MHz (vs. its absence at low field) is attributed to the maintained higher molar ratio of A5P to KDO8PS. It is the shorter data acquisition time at the high field (about 20 min) vs. that at the low field (about 7.5 h) which helps to minimize KDO8PS-phosphatase activity.

No evidence for the bound A5P-phosphate peak is present at the high field spectrum, similar to our observations for PEP at this field. The absence of the bound peak is interpreted in terms of the excessive broadening imparted to the enzyme-bound phosphate moiety. It should be noted that the bound peak is not observed also when 12,000 transients were acquired. Moreover, for PEP at 202.5 MHz also the free form was not observed. The appearance of the free peak at 202.5 MHz for A5P is attributed to a weaker binding of A5P ($K_m = 26\,\mu M$) vs. PEP ($K_m = 6\,\mu M$), leading also to a smaller fraction of A5P's bound form.

3.3. KDO8PS–KDO8P binary complex

Of the two products of the catalytic reaction, Pi and KDO8P, the latter exhibits five forms that can inter-convert via mutarotation processes [28,29], four of which are cyclic and one acyclic (Scheme 3). Also here, as for A5P, knowing which of the anomeric forms is the actual reaction product is important for unraveling the final steps of the enzymatic reaction. Earlier studies in which two synthetic cyclic analogs of the pyranose anomers, namely 2-deoxy-α- and β-pyranose KDO8P, were synthesized and kinetically characterized [11], showed that the β-pyranose analog inhibits the catalytic reaction slightly better than the α-pyranose analog (K_i values of 303 μM vs. 470 μM). Based on these observations, it was suggested that the pyranose anomers of KDO8P could be the actual forms released by the synthase (following the release of Pi). In order to directly identify which of the forms of the product can be enzyme-bound, the binary complex KDO8PS–KDO8P was subjected to both ^{31}P and ^{13}C NMR characterization.



Scheme 3. Distribution of different anomeric forms of KDO8P under neutral aqueous conditions [28].

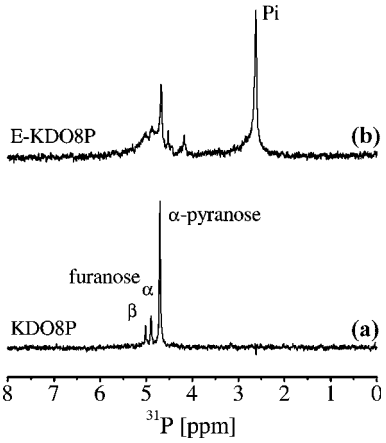


Fig. 5. 202.5 MHz ^{31}P proton-decoupled NMR spectra of: (a) KDO8P, 32 scans (20 mM in 0.1 mM Tris, pH 7.3) and (b) E-KDO8P (1:1) binary complex, 512 scans (5.3 mM KDO8PS and 5.3 mM KDO8P in 0.1 mM Tris–D_2O, pH 7.3). The peak at 4.5 ppm is unidentified; the peak at 4.2 ppm is assigned to residual A5P.

The 202.5 MHz ^{31}P NMR spectrum in Fig. 5a of KDO8P free in solution depicts resolved peaks of the three most abundant cyclic anomers [28]: β-furanose (12.0%), α-furanose (19.1%), and α-pyranose (65.8%) at 5.01, 4.87, and 4.69 ppm, respectively. In this spectrum, the peak due to the least abundant 3.1% β-pyranose anomer, is either not resolved or not detectable. In order to monitor enzyme-bound KDO8P, PEP, and A5P were enzymatically reacted by adding stoichiometric quantities to the enzyme solution, giving rise to the spectrum shown in Fig. 5b. This spectrum depicts the three free anomeric forms of KDO8P: β-furanose, α-furanose, and α-pyranose at 5.0, 4.9, and 4.7 ppm, respectively, in accordance with their free positions in solution. In addition, an unidentified peak appears at 4.5 ppm, a 4.2 ppm peak from a residual A5P, and a 2.6 ppm peak due to Pi formation during the enzymatic synthesis of KDO8P. Inspecting spectrum Fig. 5b, we note that the peaks due to the furanose forms of KDO8P are broadened, while the relative intensity of the pyranose peak is substantially reduced compared to its relative intensity in the absence of enzyme (Fig. 5a); it is therefore concluded that the peak due to the bound pyranose form is too broad to be detected (vide infra). The combination of these observations indicates that KDO8P binding to the enzyme shifts the equilibrium conditions of the free forms of its anomers, where binding of the pyranose form is preferential and tighter. The weaker binding of the furanose forms induces only limited broadening that is detectable at the high field. This interpretation of the data is further supported by the following results at the low field spectrometer.

The ^{31}P experiments were repeated at the low field spectrometer, however, this time by directly adding KDO8P to the apo-enzyme. The reference 81.0 MHz spectrum of KDO8P shown in Fig. 6a is similar to the high field spectrum shown in Fig. 5a, yet with poorer resolution. The apo-enzyme ^{31}P spectrum in Fig. 6b shows

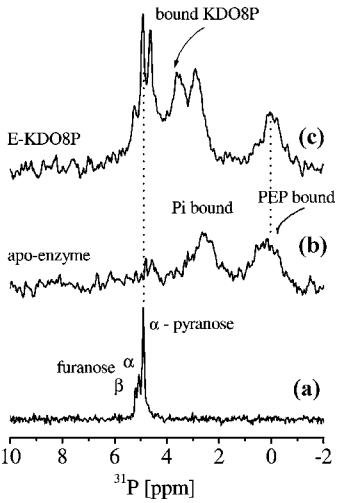


Fig. 6. ^{31}P proton-decoupled 81.0 MHz NMR spectra of: (a) KDO8P, 64 scans (20 mM in 0.1 mM Tris, pH 7.3); (b) apo-KDO8PS obtained after extensive dialysis, 19,000 scans (5.3 mM in 0.1 mM Tris–D_2O, pH 7.3); and (c) after addition of 1 equiv. KDO8P to (b), 15,000 scans. Spectra b and c are normalized to the number of scans.

two broad peaks at 2.3 and −0.3 ppm of residual quantities of enzyme-associated Pi and PEP [6,14], respectively, that could not be fully removed by the dialysis. These residual quantities are estimated to be in the range of 0.2–0.3 equivalents [30]. Addition of one equivalent of KDO8P to this apo-enzyme gives rise to the spectrum shown in Fig. 6c. In this spectrum the two sharper downfield peaks are attributed to the free forms of KDO8P in solution, β- and α-furanose unresolved at 5.0 ppm, and α-pyranose at 4.7 ppm. The narrow 4.4 ppm peak was not identified. In addition to the somewhat narrower peaks for the enzyme bound species, E-PEP and E-Pi (2.6 and −0.2, respectively), a new broad peak appears at 3.3 ppm and is attributed to enzyme-bound KDO8P. However, it should be noted that this peak itself does not identify which anomer of KDO8P is enzyme-bound.

It should be noted that the 202.5 MHz spectrum in Fig. 5b and the 81.0 MHz spectrum in Fig. 6c correspond to similar sample compositions, E/KDO8P/Pi with 1:1:1 ratio, even though they were differently obtained. The absence of a bound 3.3 ppm peak in the spectrum of Fig. 5b (while present in Fig. 6c) is attributed to the excessive broadening encountered at the high field, as was observed above for both E-PEP and E-A5P binary complexes.

In an attempt to further substantiate the above results, we have applied ^{13}C solution NMR to a 1:1 ratio of KDO8PS–[2,4-$^{13}C_2$]KDO8P binary complex. First, the 50.3 MHz proton-decoupled ^{13}C spectrum of [2,4-$^{13}C_2$]KDO8P is shown in Fig. 7a. Also here, as in the ^{31}P spectra above, peaks of the C2 and C4-labeled carbons of KDO8P due to only the three most abundant anomers are observed. Their respective chemical shifts are summarized in Table 1. Since the doubly labeled KDO8P was

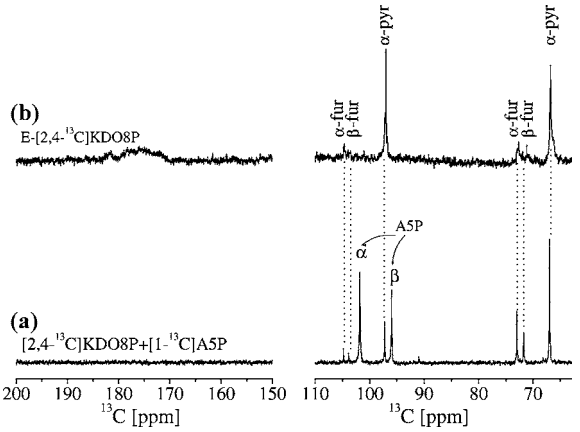


Fig. 7. ^{13}C proton-decoupled NMR spectra of: (a) [2,4-^{13}C]KDO8P, [2-^{13}C]KDO8P, and [1-^{13}C]A5P at 50.3 MHz, 256 scans (22, 10, and 14 mM, respectively, in 0.1 mM Tris–D_2O, pH 7.3; see Experimental) and (b) KDO8PS–[2,4-^{13}C]KDO8P 1:1 binary complex at 125.8 MHz, 2024 scans (conditions as in Fig. 6).

Table 1
Chemical shifts of [2,4-$^{13}C_2$]KDO8P free in solution and of its complex with the enzyme

	α-Pyranose		β-Pyranose		α-Furanose		β-Furanose	
	Free	Complex	Free	Complex	Free	Complex	Free	Complex
$C2^{KDO8P}$	96.9	97.2	97.9[a]	—	104.7	104.8	103.4	103.8
$C4^{KDO8P}$	66.8	66.9	68.0	—	72.8	72.9	71.5	71.7

[a] Assignment is based on the spectrum of [2-^{13}C]KDO8P at 50.3 MHz spectrometer (data not shown).

prepared enzymatically from [2-^{13}C]PEP and excess of [1-^{13}C]A5P, the spectrum in Fig. 7a shows also the C1 peaks of A5P's α- and β anomers. The 125.8 MHz proton-decoupled ^{13}C spectrum of the KDO8PS–[2,4-$^{13}C_2$]KDO8P binary complex (1:1 ratio) is shown in Fig. 7b. The spectrum obtained at the low field is similar, except to poorer S/N and is not shown. This spectrum reveals a pattern of peaks of the C2 and C4 carbons that are similar to those observed for the free KDO8P. It should also be noted that for the KDO8PS–KDO8P complex, the chemical shift positions are practically unaltered (within experimental error), and a small broadening of only the C4 peaks is observed.

The fact that binary complex formation of KDO8PS–KDO8P has only a negligible effect on the ^{13}C spectrum, while pronounced effects are imparted to the ^{31}P spectrum, implies that these carbons—$C2^{KDO8P}$ and $C4^{KDO8P}$, hardly sense chemical or motional effects and are, therefore, far less sensitive reporters compared to the phosphate group. This fact is rationalized knowing that the phosphate moiety is the essential one for KDO8P binding by the enzyme. This distinction is further substantiated in view of the gradual decrease in the effect on the ^{13}C spectra, starting with PEP binary complex where significant broadening was observed, through the A5P complex with minor broadening, and finally the KDO8P complex depicting

negligible line broadening. This trend is attributed to the decrease of binding affinity of the three, PEP, A5P, and last KDO8P, as well as to the fact that the binding site of KDO8P phosphate (that of A5P) is an exposed one, and, therefore, allowing for motional degrees of freedom.

4. Discussion and conclusions

Monitoring the natural substrates (PEP, A5P) and product (KDO8P) of KDO8PS as enzyme–ligand binary complexes under conditions of thermodynamic equilibrium was done both at low and high field solution NMR spectrometers, 200 and 500 MHz (^{1}H frequencies). Our ^{31}P NMR measurements show the effects imparted to the phosphate groups of the ligands upon binding. The latter is a functional moiety that plays a key role for the recognition and binding of each one of the ligands by the enzyme. As an additional probe, ^{13}C NMR measurements were applied to binary complexes specifically labeled with ^{13}C at desired positions, in particular positions that exhibit high chemical shift sensitivity to the occurring anomeric form as in A5P and KDO8P. In this simple minded NMR approach, interactions of the small ligand molecules with the 120 kDa tetrameric protein are manifested by line broadening and/or line shifts.

Our ^{31}P solution NMR measurements enabled us to identify the formation of enzyme–ligand binary complex. The ^{31}P phosphate peaks of both substrates, PEP and A5P, and product, KDO8P, are substantially broadened upon enzyme binding. Being broadened beyond detection at the high field, only the low field spectrometer is capable of providing direct experimental evidence on these interactions. Examining the chemical shift of the phosphate groups of the ligands upon binding, the following facts should be noted. A5P shifts from 4.2 to 3.8 ppm and KDO8P shifts from 4.7 to 3.3 ppm. The similarity of the chemical shifts of enzyme-bound A5P and KDO8P are attributed to the fact that both phosphates occupy the same site and, therefore, experience a similar chemical environment. However, the chemical shift of PEP remains unaltered upon binding, indicating similar electrostatic interactions of both bound and unbound forms. It should also be noted, that the extent of change of the chemical shift upon binding (bound vs. free), does not necessarily correlate with the binding strength: PEP, the most tightly bound ligand, exhibited no change of chemical shift. Line broadening is a much more sensitive and representative parameter to report on the binding strength of a small molecule to the protein.

Except for [2-^{13}C]PEP where significant line broadening was displayed upon binding, the ^{13}C spectra of both [1-^{13}C]A5P and [2,4-$^{13}C_2$]KDO8P showed only limited broadening effects at both field strengths with no line shifts. The larger effect is attributed to PEP's tighter binding. For A5P, the relatively small line broadening of the most abundant α- and β-furanose anomers, can provide only limited support that these two forms are those recognized and bound by the enzyme in accordance with our earlier solid state NMR observations. ^{31}P NMR fails to distinguish between the anomers and cannot be of help on this issue.

It is noteworthy that KDO8PS from *E. coli* studied in this work does not require metals [1], however, it was recently demonstrated that KDO8PS from the hyperthermophilic bacterium *Aquifex aeolicus* [31] and from the pathogenic bacterium *Helicobacter pylori* [32], requires divalent metal cofactor for catalysis. In addition, crystal structures of *A. aeolicus* KDO8PS complexes with various combinations of the natural substrates, A5P and PEP, and with the activating metal ion Cd^{2+} were determined [33,34]. Interestingly, in both reported structures that included A5P, KDO8PS–A5P, and KDO8PS-PEP-A5P, this sugar substrate appears in its acyclic aldehyde form. For the *E. coli* enzyme, however, our earlier solid-state NMR studies [15] and the current observations show that A5P binds to the enzyme via its cyclic forms. Understanding the origin of the differences in A5P binding between metal-dependent and metal-independent enzymes will require further investigation.

Contrary to the case for A5P, the distinction between binding affinity of the product (KDO8P) anomeric forms is successfully facilitated by ^{31}P NMR at both fields. In spite of the fact that there is no chemical shift resolution between the bound forms, the free, most abundant cyclic pyranose and furanose anomers, are well resolved and hence, enable to determine that the binding affinity of α-pyranose is the highest. The stronger binding of the pyranose vs. that of the furanose anomers, supports the notion that the cyclic pyranose anomer could be the final product of the catalytic reaction. This distinction among the anomeric forms of KDO8P is also in agreement with earlier studies, where cyclic pyranosidic analogs were synthesized and kinetically characterized [11].

In conclusion, the most significant information was obtained by monitoring the phosphate groups via ^{31}P NMR at the lower field spectrometer. At the high field, ^{31}P peaks of bound species were broadened beyond detection. The tabulation of chemical shifts of the free and bound ligands served as a reference for the solid state NMR investigation of KDO8PS [15]. Therefore, the agreement between the ^{31}P shifts of the bound species in solution with those observed by solid state NMR of lyophilized complexes, validated that in the latter, native bound forms are actually preserved. Furthermore, the solution NMR experiments allowed us to probe the state of the bound ligands together with the free ligands present in solution under thermodynamic equilibrium, and to draw a qualitative description of their relative binding strength. It should be noted that X-ray data cannot elucidate such aspects. Microcalorimetric measurements designed to determine the thermodynamic parameters were not conclusive so far due to the enormous complexity of the bio-macromolecular system. In this study, the evidence gained on the binding of the natural substrates and products themselves via solution NMR, is mechanistically insightful and complements evidence gained from the synthesis and evaluation of analogs.

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