Pergamon

*Bioorganic & Medicinal Chemistry Letters*, Vol. 7, No. 19, pp. 2463-2468, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)10021-X

# CATALYTIC MECHANISM OF KDO8P SYNTHASE. PRE-STEADY-STATE KINETIC ANALYSIS USING RAPID CHEMICAL QUENCH FLOW METHODS

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**Abstract**: The catalytic mechanism of 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (Kdo8P) synthase from *Escherichia coli* was investigated under pre-steady-state conditions using rapid chemical quench flow methods. The results suggest the formation of acyclic bisphosphate **1** as a reaction intermediate. © 1997 Elsevier Science Ltd.

3-Deoxy-D-*manno*-2-octulosonate-8-phosphate (Kdo8P) synthase (EC 4.1.2.16) is a key enzyme that controls the carbon flow in the biosynthetic formation of 3-deoxy-D-*manno*-2-octulosonate (Kdo),[1] a constituent of the lipopolysaccharide of most Gram-negative bacteria.[2] The enzyme catalyzes the unusual condensation of phosphoenolpyruvate (PEP) with D-arabinose-5-phosphate (A5P) to produce Kdo8P and inorganic phosphate ($P_i$).[3] The mechanistic details of this transformation (Scheme 1) have been the subject of considerable controversy. Although earlier studies[4] have established that the net process is essentially irreversible and involves C–O bond cleavage of PEP, there are at least two chemically feasible reaction pathways. These pathways, as illustrated in Scheme 1, involve the formation of either the acyclic bisphosphate intermediate **1** (path a),[4,5] or the cyclic bisphosphate intermediate **2** (path b).[5,6] In order to provide definitive evidence to distinguish between these possibilities, our efforts were directed toward two parallel approaches: (1) Nonenzymatic generation of the proposed cyclic intermediate **2** and its evaluation with the enzyme as alternate substrate; (2) The use of rapid chemical quench flow methods to provide direct observation and characterization of putative enzyme reaction intermediate(s). The latter approach is particularly important not only for the direct evidence for the existence of a putative reaction intermediate(s),[7] but also to provide a detailed account of the catalytic events occurring at the enzyme active site. In the preceeding paper of this issue,[8] we described our results from using the first approach which involved the evaluation of synthetic **2** with the enzyme. The results led to the conclusion that **2** is *not* a true intermediate generated during the enzyme catalysis. The present study lends further support to this conclusion. In the course of this work, we have established that the release of product (Kdo8P) from the enzyme is at least partially rate-limiting and a faster step limits chemical catalysis.

It is interesting to note, that while most enzymatic reactions utilizing PEP as a substrate involve a cleavage of the high-energy P–O bond ($\Delta G = -14.8$ kcal/mol), only four enzymes have been shown to include the unusual cleavage of the C–O bond of PEP.[9] Two of them are: UDP-GlcNAc enolpyruvoyl transferase (MurZ), an enzyme involved in peptidoglycan biosynthesis,[10] and 5-enolpyruvoyl-shikimate-3-phosphate (EPSP) synthase, which is part of the shikimate pathway.[11] These enzymes catalyze the transfer of enolpyruvoyl moiety from PEP to the respective cosubstrate alcohols and are the targets of commercially important inhibitors; MurZ is targeted by the antibiotic fosfomicin,[12] and EPSP synthase is the site of action of

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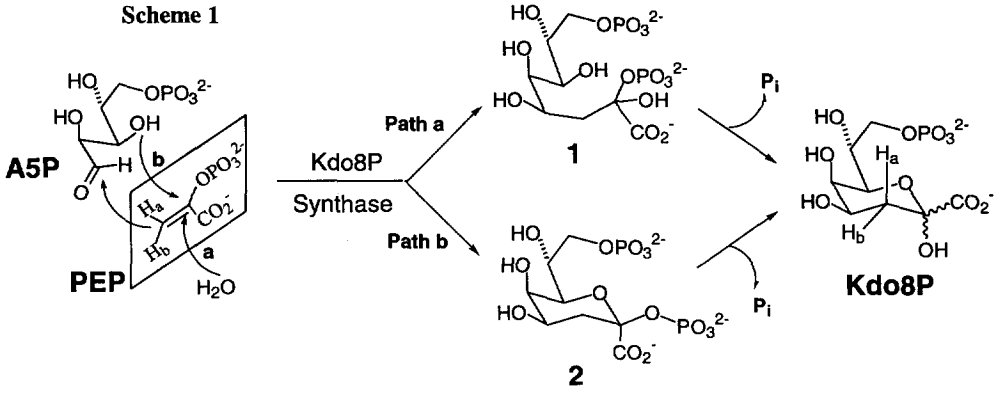
the herbicide glyphosate.[13] Two other enzymes that represent a second distinct class of enzymatic reactions involving C–O bond cleavage of PEP, catalyze the formation of a net aldol condensation product via coupling of C-3 of PEP with a cosubstrate aldehyde. This class is represented by Kdo8P synthase and 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase, an enzyme in the shikimate pathway that catalyzes a reaction between PEP and erythrose-4-phosphate to produce an unusual seven carbon sugar, DAHP.[14]

**Scheme 1**




Rapid chemical quench flow studies of EPSP synthase, performed by Anderson et al.,[15] have clearly demonstrated the formation of a single tetrahedral intermediate which is formed by an attack of the 5-OH of shikimate-3-phosphate on C-2 of PEP. A thorough kinetic and thermodynamic characterization of this reaction, including the isolation and structural determination of the reaction intermediate,[7] has provided complete confirmation of earlier proposals suggesting the formation of this intermediate. Similar studies performed on MurZ by Anderson and Walsh,[16] have led to the isolation and characterization of two kinetically competent intermediates: a covalent phospholactyl-enzyme adduct and a phospholactyl-UDP-GlcNAc tetrahedral intermediate.

While the reaction mechanisms for the two enolpyruvoyl tranferase enzymes have been unambigously determined, the reation pathways for Kdo8P synthase and DAHP synthase remain unclear. Since a detailed understanding into the nature of reaction intermediates in the enolpyruvoyl transferases has been gained through the use of transient kinetic methods, the application of these methods to the study of Kdo8P synthase should provide further insight into the catalytic mechanism of the enzyme. Since the proposed cyclic bisphosphate intermediate (**2**, Scheme 1) is very similar in functionality to the ketal phosphate intermediates isolated for MurZ and EPSP synthase, we were encouraged that this type of intermediate might be isolated and characterized for Kdo8P synthase. In this report we describe the application of these methods to investigate the catalytic mechanism of Kdo8P synthase.

*A presteady-state burst of product formation.* The examination of the events occurring at the active site of the enzyme was initiated by performing a pre-steady-state burst experiment[17] as shown in Figure 1. The time course shows the time-dependent, biphasic formation of radiolabeled product (Kdo8P). The observation of a burst of product (91 $s^{-1}$) in this experiment indicates that product release from the enzyme is at least partially rate-limiting. The rate of the slower linear phase (0.46 $s^{-1}$) corresponds to the steady-state rate ($k_{cat}$) at 15 °C, indicating that the release of product from the enzyme is the rate-limiting step in the overall reaction pathway. This result was promising in terms of allowing detection of an enzyme intermediate. Since the chemical step does not limit the overall reaction, this may indicate that an intermediate may at least transiently accumulate during a single enzyme turnover. The amplitude of the burst provides an accurate estimate of the concentration of the enzyme active sites (9 μM) which is very close to the determined protein concentration (10 μM) (90% active). It has been suggested from the preliminary X-ray crystallographic studies that the enzyme exists as a homodimer having a molecular weight of 30 kDa per monomer.[18] The data from this experiment establishes that each monomeric subunit is catalytically active.

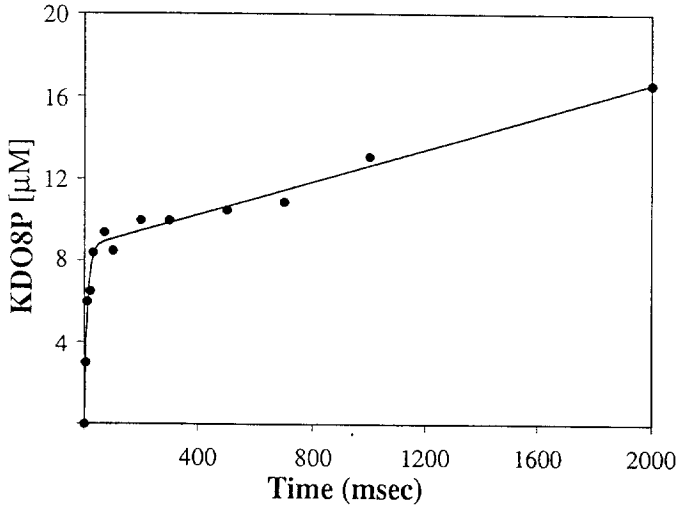



**Figure 1.** *Kinetics of a Presteady State Burst of Product in KDO8P Synthase*: A solution containing enzyme (10 μM) preincubated with [$^{14}$C]PEP (200 μM) was mixed with A5P (200 μM). The reaction was terminated by quenching with 0.2 N KOH at 15 °C. (Final concentration after mixing). The curve represents a fit to a burst equation with a rate of 91 $s^{-1}$ for the fast phase and 0.46 $s^{-1}$ for the linear phase at an amplitude of 9 μM.

*Single Turnover Experiments to Detect Enzyme Intermediates.* The most definitive experiment to examine the reactions at the active site and for the detection of reaction intermediates is termed a single turnover experiment.[7] This type of experiment involves following the conversion of substrate to product in a single enzyme turnover, with enzyme in excess over a limiting amount of radiolabeled substrate. Since enzyme is in excess over substrate, the dissociation of products is not rate-limiting and the possibility for detecting transient enzyme intermediates is optimized. We have performed single turnover rapid chemical quench experiments to examine both the forward and reverse directions in an attempt to identify and characterize a

putative enzyme intermediate. Since earler studies[5,6] suggested that the cyclic bisphosphate, **2**, may be a plausible reaction intermediate, a concentrated effort was directed toward the direct observation of this species during the course of catalysis. The preceding report[8] has shown that a synthetic sample of **2** is stable under basic conditions, with the $t_{1/2}$ at 37 °C and pD 7.0 estimated to be 2.5 h. The other plausible reaction intermediate, the acylic bisphosphate **1,** has not been synthesized but may be expected to be highly reactive since it is a hemiketal phosphate species. In each case, the conversion of radiolabeled substrate to product was examined using a method which involved anion-exchange HPLC separation with simultaneous radioactive flow detection.[19] Since we know the elution times for the enzyme substrates and products, an intermediate would be expected to have a different HPLC retention time. The reaction intermediates **1** or **2** are more highly charged and therefore would elute later than substrates or products using anion-exchange HPLC. The following set of experiments were conducted to provide evidence for an intermediate: (1) A preincubation mixture of excess enzyme and [$^{32}$P]PEP or [$^{14}$C]PEP was mixed with a saturating concentration of a unlabeled A5P; (2) A preincubation mixture of excess enzyme and a saturating concentration of unlabeled PEP was mixed with a limiting amount (relative to enzyme) of [$^{32}$P]A5P or [$^{14}$C]A5P; (3) Excess enzyme was preincubated with a limiting amount [$^{32}$P]Kdo8P or [$^{14}$C]Kdo8P and was mixed with a saturating concentration of unlabeled inorganic phosphate. In each case, the enzyme reaction was quenched at various times (0.005, 0.020, 0.100, 1, 10 sec) after mixing by the addition of 0.2 N KOH (final concentration), and the amount of radiolabeled product (Kdo8P) was quantified as described in the above experiments. We especially looked for the appearance of new peaks in the HPLC profile, which may be attributed to the bisphosphate intermediate.

In each of the experiments described above, we did not find any species that could be ascribed to the bisphosphate intermediate, **2**. In order to further evaluate the possibility of **2** as a reaction intermediate, we examined the ability of synthetic **2** to serve as an enzyme substrate under the same conditions used for the rapid chemical quench experiments. The retention time of **2,** using an analytically pure sample mixed with 0.2 N KOH (final concentration), was determined to be 40 min as determined by assaying fraction using an inorganic phosphate assay procedure.[20] The elution time for **2** is substantially higher than the retention times of substrates (12 min and 32 min for A5P and PEP, respectively) and product (Kdo8P, 21 min). This result provides evidence that if **2** was a true reaction intermediate, we should have detected it under the experimental conditions used.

In order to examine the possibility of formation of an enzyme-bound covalent intermediate, the quenched reaction mixtures in which either [$^{32}$P] labeled A5P, PEP, or Kdo8P was employed, were also subjected to SDS-page analysis in conjunction with radioactivity detection by phosphorimaging. No radiolabel was associated with the band of the enzyme (~30 kDa) indicating that no enzyme-linked intermediate is formed during the catalysis. Therefore the nucleophile which attacks PEP might be an activated water molecule rather than an amino acid residue at the active site of the enzyme.

In summary, this work and the preceding paper have examined the mechanistic pathway for the reaction catalyzed by Kdo8P synthase and have tested the hypothesis that the cyclic bisphosphate, **2**, is a reaction intermediate. Two parallel approaches were involving in examining whether **2** is an enzyme intermediate in the catalytic pathway: (1) Chemical synthesis of **2** and evaluation as an alternate substrate for the enzyme; (2) Rapid chemical quench flow studies to provide direct observation and characterization of the putative intermediate during enzyme catalysis. The results of first approach have demonstrated that the synthetic **2** is

neither a substrate nor a potent inhibitor of the enzyme. Conceivably, the failure of **2** to serve as an alternate substrate maybe related to the fact that when **2** binds to enzyme, this represents a step which is not normally on the pathway of the reaction. Accordingly, the observed weak binding ($K_i$ = 35 mM) may result from a kinetic barrier must be overcome in which the **2** binds to a rare conformation of enzyme or induces a slow change in conformation after formation of an initial weak collison complex.[21]

This possibility was ruled out by the rapid chemical quench flow experiments that clearly demonstrate that the cyclic bisphosphate intermediate, **2**, does not accumulate under single enzyme turnover conditions. This observation, coupled with our parallel results obtained through the evaluation of synthetic **2** as a substrate of the enzyme, strongly suggest that the Kdo8P synthase-catalyzed reaction does not involve the formation of 2 as a reaction intermediate. A remote possibility is that the failure to observe the cyclic bisphosphate species may, in principle, be precluded by unfavorable reaction kinetics in which the radiolabeled intermediate does not accumulate to a significant level to allow detection. This suggestion is countered by our observations; the fact that **2** is not an enzyme substrate but rather an inhibitor indicates that this is not the case. Taken together, these results support the original hypothesis of Hedstrom and Abeles,[4] which suggests a reaction pathway involving an acyclic bisphosphate intermediate (**1**, Scheme 1). Experiments are underway to provide a complete kinetic and thermodynamic description of the reaction pathway including a more detailed study to identify and characterize the true reaction intermediate or its decomposition products.

**Acknowledgments**: We are grateful to Shoucheng Du and Dr. Valery Belakhov for help and advice. This research was supported by the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel (Grant No. 94-00371) and National Institute of Health GM 55431 to KSA.

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17. Rapid quench experiments were performed using a Kintek RFQ-3 Rapid Chemical Quench (Kintek Instruments, State College, PA) as previously described.[15,16] Briefly, the reaction was initiated by mixing the enzyme solution (15 μL) with the radiolabeled substrate (15 μL). In all cases, the concentrations of enzyme and substrate cited in the text are those after mixing and during the enzymatic reaction. The reaction mixture was then quenched with 0.6 N KOH (67 μL). Substrates and products are stable under these conditions. The substrates and products were separated and quantified using anion-exchange HPLC chromatography in combination simultaneous radioactivity detection. The HPLC separation was performed on a Mono-Q (HR 5/5) anion exchange column with a flow rate of 1 mL/min. A gradient separation was employed where solvent A is 20 mM triethylammonium bicarbonate (pH 9) and solvent B is 1 M triethylammonium bicarbonate (pH 9). The linear gradient program was as follows: 0–1 min 100% A, 1–25 min 0–25% B, 25–35 min 25–50% B, 35–45 min 50–100% B, 50–55 min 100% B followed by re-equilibration. The elution times were A5P ( 12 min), PEP ( 32 min), Kdo8P (21 min), and $P_i$ (8 min). These conditions were used to analyze samples generated from the rapid chemical quench experiments.

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(*Received in USA* 14 *July* 1997; *accepted* 29 *August* 1997)