Catalytic Mechanism of Kdo8P Synthase: Transient Kinetic Studies and Evaluation of a Putative Reaction Intermediate †

ARTICLE *in* BIOCHEMISTRY · DECEMBER 1998

Impact Factor: 3.02 · DOI: 10.1021/bi981374w · Source: PubMed

7 AUTHORS, INCLUDING:

po-huang Liang
Academia Sinica
124 PUBLICATIONS **3,380** CITATIONS
SEE PROFILE

Karen S Anderson
Yale University
194 PUBLICATIONS **5,752** CITATIONS
SEE PROFILE

Amnon Kohen
University of Iowa
125 PUBLICATIONS **2,981** CITATIONS
SEE PROFILE

Catalytic Mechanism of Kdo8P Synthase: Transient Kinetic Studies and Evaluation of a Putative Reaction Intermediate†

Po-Huang Liang,‡ Julian Lewis, and Karen S. Anderson*

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06520-8066

Amnon Kohen,§ Francis Wallace D'Souza,|| Yaakov Benenson, and Timor Baasov*

Department of Chemistry, Technion-Israel Institute of Technology, Haifa 32000, Israel

Received June 10, 1998; Revised Manuscript Received September 24, 1998

ABSTRACT: The mechanistic pathway for the reaction catalyzed by Kdo8P synthase has been investigated, and the cyclic bisphosphate **2** has been examined as a putative reaction intermediate. Two parallel approaches were used: (1) chemical synthesis of **2** and evaluation as an alternate substrate for the enzyme and (2) transient kinetic studies using rapid chemical quench methodology to provide direct observation and characterization of putative intermediate(s) during enzyme catalysis. The putative cyclic bisphosphate intermediate **2**, possessing the stereochemistry of the β-pyranose form, was synthesized and evaluated as a substrate and as an inhibitor of Kdo8P synthase. The substrate activity was examined by monitoring the release of anomeric phosphate over time using proton-decoupled ^{31}P NMR spectroscopy. A very similar time course for the formation of inorganic phosphate was found in each experiment and the corresponding control experiment; i.e., no enzyme-catalyzed acceleration in the anomeric phosphate hydrolysis was detected. It was found however that **2** binds to the enzyme and is a competitive inhibitor with respect to phosphoenolpyruvate binding, having a K_i value of 35 μM. In a parallel study, we have performed single-turnover rapid chemical quench experiments to examine both the forward and reverse directions to identify a putative enzyme intermediate(s). Our results clearly demonstrate that the cyclic bisphosphate intermediate **2** does not accumulate under single-enzyme turnover conditions. This observation, coupled with the results obtained through the evaluation of synthetic **2** as a substrate, strongly suggests that the Kdo8P synthase catalytic pathway does not involve the formation of **2** as a reaction intermediate. Taken together, these combined results support the original hypothesis [Hedstrom, L., and Abeles, R. H. (1988) *Biochem. Biophys. Res. Commun. 157*, 816−820], which suggests a reaction pathway involving an acyclic bisphosphate intermediate **1**.

Phosphoenolpyruvate (PEP)[1] is a highly functionalized, chemically versatile molecule used at several intersections of cellular energy metabolism and biosynthesis (*1*). While most enzymatic reactions utilizing PEP as a substrate involve cleavage of the high-energy P−O bond ($\Delta G^{\circ\prime} = -14.8$ kcal/mol), two types of reactions have been shown to involve the unusual cleavage of the C−O bond of PEP (*2*): (1) formation of an enol ether linkage through transfer of the enolpyruvoyl moiety in PEP to a cosubstrate alcohol (Scheme 1, path a) and (2) formation of the net aldol condensation product through coupling of C-3 of PEP with a cosubstrate aldehyde (Scheme 1, path b).

The a-type reaction pathway in Scheme 1 is represented by UDP-GlcNAc enolpyruvoyl transferase (*Mur Z*; also called *Mur A*), an enzyme involved in peptidoglycan biosynthesis (*3*) and 5-enolpyruvoylshikimate 3-phosphate (EPSP) synthase, an enzyme in the shikimate pathway involved in aromatic acid biosynthesis (*4*). These enzymes catalyze the transfer of the enolpyruvoyl moiety from PEP to respective cosubstrate alcohols and are the targets of commercially important inhibitors; MurZ is targeted by the antibiotic fosfomicin (*5*), while EPSP synthase is the site of action for the herbicide glyphosate (*6*).

Mechanistic studies on EPSP synthase using a transient kinetic approach 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 (*7*−*9*). A thorough kinetic and thermodynamic characterization of the reaction pathway, including the isolation and structural determination of a kinetically competent reaction

† This work was supported by NIH Grant GM55431 to K.S.A. and by the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel (Grant 94-00371), to T.B.

* To whom correspondence should be addressed. E-mail: karen.anderson@yale.edu. Phone: (203) 785-4526. Fax: (203) 785-7670.

‡ Present address: Department of Macromolecular Science, SmithKline Beecham, 709 Swedeland Rd., King of Prussia, PA 19406.

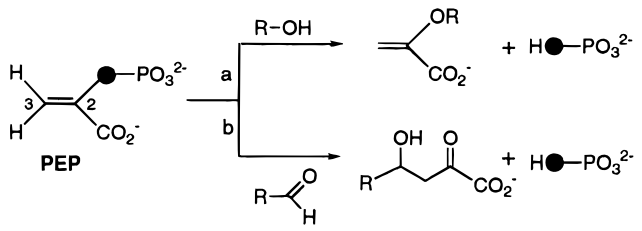
§ Present address: Department of Chemistry, University of California, Berkeley, CA 94720.

|| Present address: Department of Chemistry, The Ohio State University, 100 West 18th Ave., Columbus, OH 43210-1173.

[1] Abbreviations: A5P, D-arabinose 5-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DTT, dithiothreitol; EPSP, 5-enolpyruvoylshikimate 3-phosphate; HPLC, high-performance liquid chromatography; Mur Z, UDP-GlcNAc enolpyruvoyl transferase; Kdo8P, 3-deoxy-D-*manno*-2-octulosonic acid 8-phosphate; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)-aminomethane; UDP-GlcNAc, uridine diphospho-*N*-acetyl-D-glucosamine.

10.1021/bi981374w CCC: $15.00
Published on Web 10/29/1998

Scheme 1: Two Types of Pathways for PEP-Utilizing Enzymes Undergoing C−O Bond Cleavage



intermediate (*10*, *11*), provides strong support for a mechanism involving a single intermediate, although recently an alternate but less plausible pathway has been proposed (*12*).

A similar approach for mechanistic studies on *Mur Z* (*13*, *14*) has led to the isolation and characterization of two kinetically competent intermediates: a covalent phospholactoyl−enzyme adduct and a phospholactoyl−UDP-GlcNAc tetrahedral intermediate. The formation of a covalent phospholactoyl−enzyme adduct is a major distinction between the mechanisms of catalysis by MurZ and EPSP synthase. Nonetheless, the structural and functional homologies are suggestive of common mechanistic features, as evidenced by the isolation of similar tetrahedral intermediates in both enzyme reactions.

There are two known enzymes which can carry out the b-type reaction illustrated in Scheme 1. The first enzyme, 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (Kdo8P) synthase, catalyzes the formation of Kdo8P from D-arabinose-5-phosphate (A5P) and PEP (Scheme 2). This is an important enzymatic reaction that controls the carbon flow in the biosynthetic formation of an unusual eight-carbon sugar 3-deoxy-D-*manno*-2-octulosonate (Kdo). The Kdo is an important constituent of the lipopolysaccharide of most Gram-negative bacteria (*15*) and plays a crucial role in the assembly process of lipopolysaccharides (*15*, *16*). The second enzyme, DAHP synthase, catalyzes a similar net aldol reaction between PEP and erythrose-4-phosphate to produce 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP), and is also found in the shikimate pathway (*2*).

While the mechanisms of a-type C−O bond cleavage in two enolpyruvoyl transferase enzymes (Scheme 1, path a) have been unambiguously characterized, the mechanisms of Kdo8P synthase and DAHP synthase that represent the second distinct class (Scheme 1, path b) of enzymatic reactions involving C−O bond cleavage of PEP and net aldol condensation continue to be uncertain. Although earlier (*17*) and more recent (*18*, *19*) studies have established exclusively that the Kdo8P synthase reaction proceeds with C−O bond cleavage of PEP, the elementary steps of this transformation remain unclear.

The cloning and overexpression of the *Escherichia coli* gene encoding Kdo8P synthase activity have greatly facilitated mechanistic studies on the enzyme (*19*−*21*). Previous studies have suggested that the enzyme acts upon the acyclic form of A5P, and have demonstrated an ordered sequence of substrate binding (PEP followed by A5P) and product release (P_i prior to Kdo8P) (*22*). By using stereospecifically labeled 3-deuterio and 3-fluoro analogues of PEP as alternate substrates of Kdo8P synthase, it has been shown that the condensation step is stereospecific, involving the attachment of the *si* face of PEP to the *re* face of the carbonyl of A5P (*18*, *23*). On the basis of these mechanistic data, in combination with the results accumulated through the synthesis and examination of various analogues of A5P (*22*, *24*), analogues of PEP (*18*, *25*, *26*), and the product Kdo8P (*20*, *25*, *27*) as mechanistic probes, two distinct, chemically feasible reaction pathways have been proposed for the reaction of Kdo8P synthase. These pathways, as illustrated in Scheme 2, involve the formation of either the acyclic bisphosphate intermediate **1** (path a) (*17*) or the cyclic bisphosphate intermediate **2** (path b) (*19*, *20*).

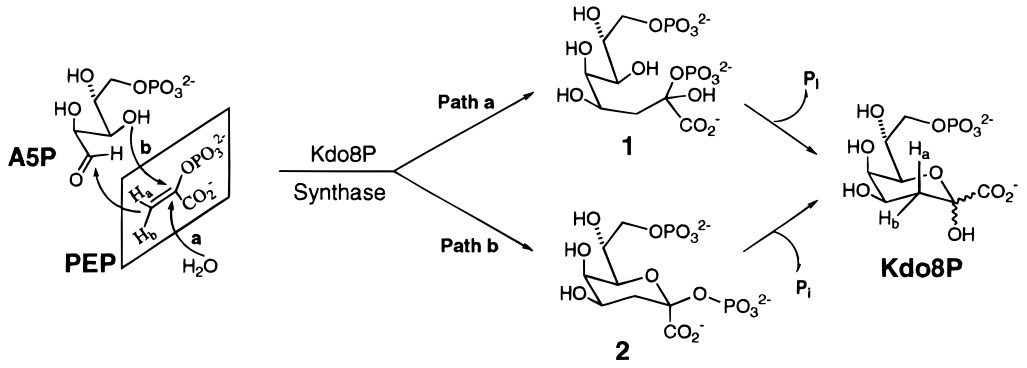
To provide definitive evidence to distinguish between these possibilities, our efforts were directed toward two parallel approaches: (1) nonenzymatic generation of the proposed cyclic intermediate **2** and evaluation of its ability to serve as a substrate for Kdo8P synthase and (2) use of rapid chemical quench flow methods to provide direct observation and characterization of putative enzyme reaction intermediate(s). The latter approach is important not only to provide direct evidence for the existence of a putative reaction intermediate(s) (*9*) but also to provide a detailed account of the catalytic events occurring at the enzyme active site. Preliminary studies have provided an initial examination of these approaches for understanding the mechanism of enzyme catalysis for Kdo8P synthase (*28*, *29*). In this report, we provide an in-depth transient kinetic analysis for characterizing the elementary steps in the enzyme reaction pathway as well as a complete description of the synthesis and evaluation of the cyclic bisphosphate intermediate **2** as an enzyme reaction intermediate. The results of both the kinetic and synthetic approaches are described, and the implications to the catalytic mechanism are discussed.

EXPERIMENTAL PROCEDURES

General Methods. The homogeneous Kdo8P synthase (specific catalytic activity of 9 units/mg) was isolated from the overproducing strain *E. coli* DH5α (pJU1), as previously described (*22*). In this study, we found that the isolated Kdo8P synthase contained an almost stoichiometric amount of PEP which was difficult to remove even with extensive dialysis. For those experiments which required the entirely PEP-free enzyme, the PEP was completely removed by incubating the enzyme with excess A5P (to convert all the tightly associated PEP to the product Kdo8P) followed by Sephadex G-25 gel filtration chromatography or through repetitive centricon ultrafiltration to remove any bound substrate or product. This purified form of Kdo8P synthase was used in single-turnover experiments to look for enzyme intermediates as described below. The enzyme concentration was determined according to the subunit molecular mass of 30 kDa (i.e., 1 μM enzyme is 0.03 mg/mL) as previously described (*29*).

For those single-turnover experiments requiring radiolabeled PEP bound to enzyme, the following procedure was necessary for the PEP to bind tightly. PEP-free enzyme (1000 μM) and radiolabeled PEP (1000 μM) were mixed in buffer [50 mM Tris-HCl (pH 7.5) containing 10 mM phosphate and 2 mM DTT], and the mixture was incubated at 4 °C overnight. The mixture was then subjected to repeated ultrafiltration through a centricon to remove unbound substrate, washed several times with buffer containing no phosphate, and concentrated to 500 μM. We found,

Scheme 2: Proposed Mechanistic Pathways for the Reaction Catalyzed by Kdo8P Synthase



however, that the enzyme was less stable when the tightly bound PEP was removed as indicated by a lower burst amplitude in pre-steady-state burst experiments. This may be related to a conformational stability of the enzyme in the presence of PEP; however, further work will be required to fully understand the issue.

The [^{14}C]A5P (55 mCi/mmol) and [^{32}P]A5P were prepared enzymatically by using hexokinase (Sigma) and [^{14}C]-arabinose (55 mCi/mmol, American Radiolabeled Chemicals Inc.) and [γ-^{32}P]ATP (Amersham), respectively. The [^{32}P]-PEP was synthesized from [^{32}P]P_i (Dupont-NEN) and EPSP synthase by using the reverse reaction. The [^{14}C]PEP (11.1 mCi/mmol) was prepared from [^{14}C]pyruvate (11.1 mCi/mmol, Amersham Co.) using pyruvate phosphate dikinase (PPDK) obtained as a generous gift from D. Dunaway-Mariano. For the examination of the Kdo8P synthase reaction in the reverse direction, [^{14}C]Kdo8P and [^{32}P]Kdo8P were also prepared enzymatically. The buffer in all cases was 50 mM Tris-HCl (pH 7.5) containing 2 mM DTT.

A5P and Kdo8P were prepared enzymatically according to the procedure of Whitesides (*30*). The potassium salt of PEP was prepared in large quantities as already described (*31*). All the chemicals used for the preparation of bisphosphate **2** were purchased from Aldrich or from Sigma and were used without further purification. Since the synthesis of **2** has recently been described (*28*), the complete synthetic scheme for the preparation of compound **2**, the detailed synthetic procedures with spectral and analytical characterization of all the intermediates and of the final product, is available as Supporting Information.

Rapid Chemical Quench Experiments. Rapid quench experiments were performed using a Kintek RFQ-3 Rapid Chemical Quench (Kintek Instruments, State College, PA) as previously described (*32*). 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 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 in combination with 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: 100% A from 0 to 1 min, 0 to 25% B from 1 to 25 min, 25 to 50% B from 25 to 35 min, 50 to 100% B from 35 to 45 min, and 100% B from 50 to 55 min, followed by re-equilibration. The elution times were 12 min for A5P, 32 min for PEP, 21 min for Kdo8P, and 8 min for P_i. These conditions were used to analyze samples generated from the rapid chemical quench experiments.

SDS−PAGE Experiments. The rapid quench experiments designed to look for covalent enzyme intermediates typically required electrophoresis of part of the quenched reaction mixture (30 μL). Glycerol (5%) and bromophenol blue (0.01%) were added to facilitate gel loading onto 15% polyacrylamide gel (Bio-Rad) using the standard Laemmli buffer system. Quantitation of the phosphor images (using a Bio-Rad molecular imager system, Hercules, CA) of the gels allowed determination of the relative amounts of radioactivity associated with Kdo8P synthase (bound) and the dye front (free). Molar concentrations were then calculated on the basis of the distribution of counts arising from the concentration of [^{32}P]PEP present in the incubation mix prior to quenching.

Substrate Trapping Experiments. To address the order of substrate binding, a limiting amount of one radiolabeled substrate is preincubated with enzyme and then mixed with a solution containing a high concentration of both cold substrates. If the radiolabeled substrate is bound in a mode which is kinetically competent, substrate will be "trapped" and radiolabeled product will be observed. Accordingly, two parallel experiments were carried out. In one experiment, a solution containing enzyme (5 μM) preincubated with [^{14}C]-PEP (25 μM) was mixed with a solution of unlabeled PEP (1 mM) and A5P (1 mM). The reaction was terminated by addition of 0.2 N KOH, and the formation of radiolabeled Kdo8P was quantified by HPLC with radioactivity detection. In a parallel experiment, a solution containing enzyme (5 μM) preincubated with [^{14}C]A5P (25 μM) was mixed with a solution of unlabeled PEP (1 mM) and A5P (1 mM) and the formation of radiolabeled Kdo8P was monitored as described above.

*Examination of **2** as an Intermediate of Kdo8P Synthase-Catalyzed Reaction.* To examine the interaction of **2** with the synthase, a proton-decoupled ^{31}P NMR assay in which the appearance of inorganic phosphate can be clearly monitored was used. Reaction mixtures contained 0.1 M Tris-DCl buffer (prepared in D_2O at pD 7.0), bovine serum albumin (1.5 mg/mL, for the stabilization of the enzyme), 5 mM substrate (compound **2**), A5P (1 mM), and 72 units of Kdo8P synthase (approximately 8 mg) in a total volume of

0.6 mL. A control experiment, containing all of the above, but without the enzyme and A5P, was run parallel to the above. The release of anomeric phosphate was monitored over time via proton-decoupled ^{31}P NMR at constant temperatures (separate experiments at 25 and 37 °C). The anomeric phosphate resonance of **2** (−1.7 ppm) gradually diminished in intensity with time and was replaced by a new singlet at δ (2.6 ppm) of inorganic phosphate, which was further identified by the addition of an authentic sample of inorganic phosphate. A very similar time course for the formation of inorganic phosphate was determined in each experiment and the corresponding control experiment, as judged by the integration of signals. A comparison of the spectra of compound **2** in the presence and absence of enzyme failed to show any significant enzyme-catalyzed acceleration in the anomeric phosphate hydrolysis after 24 h. Nonetheless, in this way the half-lives for the hydrolysis of **2** at 25 (4.7 h) and 37 °C (2.5 h) could be determined. PEP-free enzyme was not used for the NMR experiments since the enzyme is much less stable in this form and loses activity. The presence of A5P (K_m = 25 μM) or residual Kdo8P (K_i = 0.6 mM) should not influence the binding of the **2** (K_i = 35 μM) that was at a concentration 5-fold higher (5 mM) than that of A5P (1 mM).

Spectral Methods. Spectrophotometric measurements were taken 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. ^{1}H NMR spectra were recorded on a Bruker AM-200 or AM-400 spectrometer, and chemical shifts are reported (in parts per million) relative to internal tetramethylsilane (δ = 0.0) with $CDCl_3$ as the solvent and relative to HOD (δ = 4.63) with D_2O as the solvent. ^{13}C NMR spectra were recorded on a Bruker AM-200 (50.3 MHz) or AM-400 (100.6 MHz) spectrometer, and the chemical shifts are reported (in parts per million) relative to external sodium 2,2-dimethyl-2-silapentane sulfonate (δ = 0.0) in D_2O. ^{31}P NMR spectra were recorded on a Bruker AM-200 spectrometer at 81.0 MHz, and the chemical shifts are reported (in parts per million) relative to external orthophosphoric acid (δ = 0.0) in D_2O. All values for the coupling constants (J) are in hertz. Mass spectra were obtained by using a TSQ-70B mass spectrometer (Finnigan Mat) using fast-atom bombardment (FAB) in glycerol matrixes.

Enzyme Assays. Unless otherwise stated, the enzyme activity was assayed in 1.0 mL of a reaction buffer consisting of 0.1 M Tris-HCl (pH 7.0), PEP (0.2 mM, 27K_m), and A5P (0.5 mM, 20K_m). Following equilibration at 37 °C for 2 min, Kdo8P synthase (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 an internal reference) was monitored as a function of time (MS-DOS UV/VIS software). This method (*33*) is based on the absorbance difference at 232 nm between PEP (ϵ = 2840 M^{-1} cm^{-1}) and the other substrates and products (ϵ < 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. The concentrations of PEP, A5P, and **2** were determined precisely, by quantitative assaying of the P_i released by alkaline phosphatase (*34*). In each case, to ensure complete hydrolysis of the phosphate monoester, the aliquots of the incubation mixture with alkaline phosphatase were tested by ^{31}P NMR. For the determination of **2**, we found that treatment with alkaline phosphatase was not needed, as the acidity of the assay mixture was enough to bring about the complete hydrolysis of anomeric phosphate. One unit of the enzyme activity is defined as the amount that catalyzes the consumption of 1 μmol of PEP per minute at 37 °C.

Inhibition Study. To determine the steady-state kinetic parameters, K_m for PEP and A5P and K_i of **2**, the reaction solutions were prepared as described above but with a constant (500 μM, 20K_m) A5P concentration and variable (8−40 μM) PEP concentrations. The rate measurements were taken as described above, while a 5 s delay was allowed following the initiation of the reaction. The initial rate was then determined by a least-squares fitting of the first 10% of the progress curve (between 10 and 60 s, depending on the initial concentration of PEP) to a straight line. The K_m for PEP was 6 μM, and the K_m for A5P was 25 μM (*22*).

Four inhibitor concentrations were examined, and for each inhibitor concentration, four concentrations of PEP (8−40 μM) were used (Figure 6). All samples were assayed in triplicate, and analogous results were obtained in two to four different experiments. The data were fitted to the competitive model using the equation $Y = V[S]/[K(1 + [I]/K_i) + [S]]$, employing the commercial software GraFit program (*35*). The K_i value was calculated either from the above treatment or from the secondary replots of the slopes from initial double-reciprocal plots ($1/v$ vs $1/[S]$) versus inhibitor concentration (*36*).

RESULTS

Kdo8P Synthase Is Isolated with Tightly Associated PEP. Previous studies have shown that the UDP-GlcNAc enolpyruvoyl transferase enzyme is isolated with 1 equiv of covalently bound PEP in the form of a phospholactoyl species (*14*). To determine whether the Kdo8P synthase retained any tightly bound substrates after purification, the enzyme was preincubated with either radiolabeled A5P or PEP in the absence of the second substrate. We were surprised to find that the Kdo8P synthase enzyme is also isolated with 1 equiv of bound PEP. We have characterized this interaction of PEP with enzyme using NMR, SDS−PAGE protein gel analysis, and gel filtration methods to ascertain whether PEP is covalently bound to enzyme or merely tightly associated. The spectral and analytical analysis as well as rapid chemical quench experiments indicates that the PEP is *not* covalently bound but rather tightly bound in a Michaelis enzyme complex. A tightly bound form of PEP would be consistent with previous studies which have shown that PEP and A5P are condensed in a stereospecific manner to form Kdo8P and inconsistent with a pathway involving a covalent phospholactoyl species which would proceed with the loss of stereoselectivity. This was an important observation since the presence of bound PEP could complicate interpretation of rapid chemical quench results particularly in the case of single-enzyme turnover experiments. The PEP could be removed by incubating the enzyme with an excess of A5P followed by ultrafiltration to separate excess substrates and products from free enzyme (without PEP bound) for further studies. In experiments where the bound PEP was removed and replaced by radiolabeled PEP, we found it necessary to

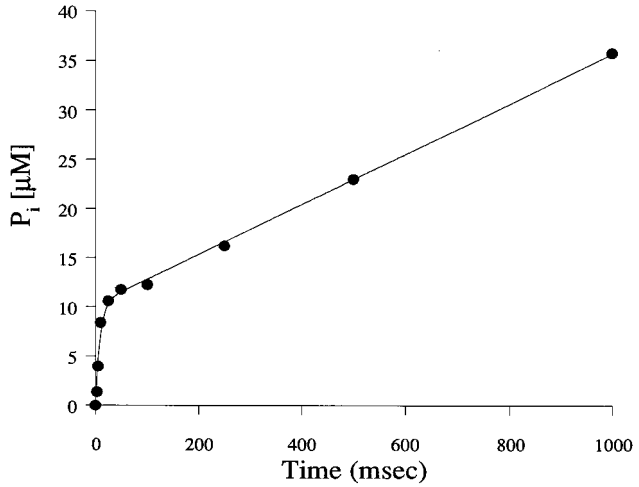


FIGURE 1: Kinetics of a pre-steady-state burst of product (P_i) in Kdo8P synthase. A solution containing enzyme (20 μM) preincubated with [^{32}P]PEP (60 μM) was mixed with A5P (500 μM) at 25 °C (final concentrations after mixing). The reaction was terminated by quenching with 0.2 N KOH, and the formation of product, P_i (●), was monitored by HPLC with radioactivity detection. The curve represents a fit to a burst equation with a rate of 110 s^{-1} for the fast phase and 2.5 s^{-1} for the slower linear phase.

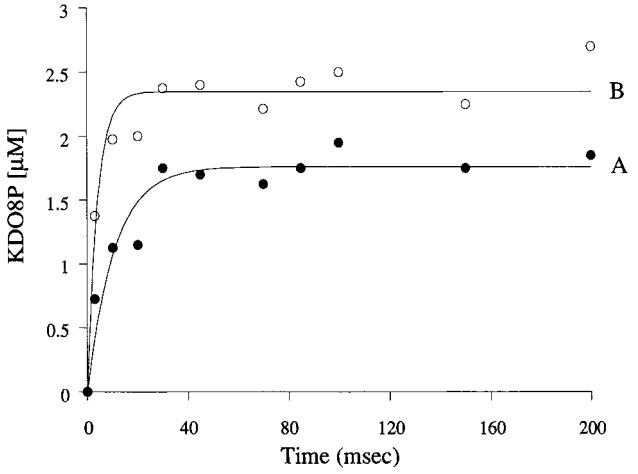


FIGURE 2: Substrate trapping in Kdo8P synthase. (A) The time course of PEP (●) trapped at the enzyme active site. A solution containing enzyme (5 μM) preincubated with [^{14}C]PEP (25 μM) was mixed with a solution of unlabeled PEP (1 mM) and A5P (1 mM). After various times, the reaction was terminated by the addition of 0.2 N KOH, and the formation of radiolabeled Kdo8P was quantified by HPLC with radioactivity detection. (B) In a parallel experiment, the time course of A5P (○) trapped at the enzyme active site. A solution containing enzyme (5 μM) preincubated with [^{14}C]A5P (25 μM) was mixed with a solution of unlabeled PEP (1 mM) and A5P (1 mM) (final concentrations), and the formation of radiolabeled Kdo8P was monitored as described above. Similar amounts of Kdo8P product were trapped using PEP (1.7 μM) and A5P (2.3 μM), indicating that the order is random such that either PEP or A5P can bind first in a mode kinetically competent for catalysis.

add phosphate to the buffer for the PEP to bind tightly (see Experimental Procedures).

A Pre-Steady-State Burst of Product Formation. We have previously shown in a rapid chemical quench experiment performed under pre-steady-state burst conditions using [^{14}C]-PEP (*29*) that there is a biphasic formation of Kdo8P product. In this study, we have conducted similar pre-steady-state burst experiments using [^{32}P]PEP, [^{14}C]A5P, or [^{32}P]A5P as substrates to determine the rate of formation of radiolabeled products: [^{32}P]P_i, [^{14}C]Kdo8P, and [^{32}P]Kdo8P, respectively. A representative burst experiment showing the time course for biphasic formation of P_i is shown in Figure 1. Similar results were obtained regardless of the radiolabeled substrate employed in the experiments. In each case, the rate of product formation was approximately 110 s^{-1} for the fast phase and 2.5 s^{-1} for the slower linear phase which corresponds to the steady-state rate (k_{cat}) at 25 °C. The amplitude of the burst experiment shown in Figure 1 provides an estimate of active site concentration which was determined in this case to be approximately 50%. We have found that, in general, the enzyme is less stable when PEP is removed. Thus, in the preparation of the enzyme for experiments using radiolabeled PEP, the tightly bound cold PEP must first be removed, often resulting in a loss of activity and a lower concentration of active sites. In separate pre-steady-state burst experiments, particularly when using radiolabeled A5P (where removal of PEP is not necessary), the amplitude is closer to 100%, indicating that almost all the enzyme sites are active (*29*).

Previous steady-state kinetic studies (*22*) have shown that the release of product from the enzyme is ordered with P_i being released first followed by Kdo8P. Since we observed a burst with either P_i or Kdo8P, this indicates that the release of product Kdo8P from the enzyme is the rate-limiting step in the overall reaction pathway.

Substrate Trapping Experiment. Previous steady-state results have suggested that the Kdo8P synthase reaction is ordered with PEP binding first followed by A5P (*22*). We performed a substrate trapping experiment by preincubating enzyme with either radiolabeled A5P or PEP. Surprisingly, the substrate trapping experiment indicated that either the PEP or A5P could bind in mode which was kinetically competent to undergo catalysis as illustrated in Figure 2. Although either substrate can form a complex which can promote catalysis, there may be a preference for PEP which is related to enzyme stability. This is consistent with our observations that the enzyme is isolated with 1 equiv of tightly bound PEP and is less stable when the PEP is removed. Moreover, the cellular concentration for PEP (estimated to be 90−100 μM) is likely to be higher than for A5P.

Single-Turnover Experiments To Look for Enzyme Intermediates. We have previously shown for several enzyme systems that the most definitive experiment for examining the reactions at the active site of the enzyme and detecting enzyme intermediates is a single-enzyme turnover experiment (*9*, *13*, *14*, *32*). 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, thus optimizing the possibility for detecting transient enzyme intermediates. We have extensively looked for enzyme intermediates using single-turnover experiments as summarized in Table 1. These single-turnover rapid chemical quench experiments, aimed at trapping intermediates, have been conducted using radiolabeled substrates in the forward direction and radiolabeled products in the reverse direction. In the forward direction, four sets of experiments (a−d) were conducted in which the radiolabel was placed in either the

Table 1: Single turnover experiments to look for intermediates

conditions[a]	analysis (product detection) HPLC	SDS−PAGE	centricon
(1) forward reaction			
(a) enzyme with [^{32}P]PEP and A5P (sat.)	[^{32}P]P_i	*b*	−
(b) enzyme with [^{14}C]PEP and A5P (sat.)	[^{14}C]Kdo8P	−	*b*
(c) enzyme with [^{32}P]A5P and PEP (sat.)	[^{32}P]Kdo8P	*b*	−
(d) enzyme with [^{14}C]A5P and PEP (sat.)	[^{14}C]Kdo8P	−	*b*
(2) reverse reaction			
(a) enzyme with [^{32}P]Kdo8P and P_1 (sat.)	*c*	*b*	−
(b) enzyme with [^{14}C]Kdo8P and P_i (sat.)	*c*	−	*b*
(c) enzyme with Kdo8P and [^{32}P]P_i (sat.)	*c*	*b*	−

[a] In all experiments, the enzyme was in excess over the radiolabeled substrate. Time courses were examined from 5 ms to 10 s. [b] No covalent enzyme species was detected. [c] No radiolabeled products (A5P or PEP) were detected in the reverse direction.

PEP or A5P substrate. The experiments were carried out as follows. Excess enzyme which has been preincubated with a limiting amount of (a) ^{32}P-labeled PEP or (b) ^{14}C-labeled PEP was mixed with a saturating amount of cold A5P, and excess enzyme which has been preincubated with a limiting amount of (c) ^{32}P-labeled A5P or (d) ^{14}C-labeled A5P was mixed with a saturating amount of cold PEP.

In the reverse direction, three sets of experiments (a−c) were conducted in which the radiolabel was placed in either the Kdo8P or P_i. The experimental conditions were as follows. Excess enzyme which has been preincubated with a limiting amount of (a) ^{32}P-labeled Kdo8P or (b) ^{14}C-labeled Kdo8P was mixed with a saturating amount of cold P_i, and (c) excess enzyme which has been preincubated with a limiting amount of unlabeled Kdo8P was mixed with a high concentration of [^{32}P]P_i. Time courses were examined from 5 ms to 10 s. HPLC analysis was used to quantitate the conversion of substrate to product as well as to identify new radiolabeled peaks which might represent noncovalently bound enzyme intermediates such as the cyclic bisphosphate, **2**. This analysis was coupled with SDS−PAGE protein gel analysis and phosphorimaging to identify covalently bound enzyme intermediates which may contain the ^{32}P moiety. For experiments in which the radiolabel employed was the ^{14}C moiety, we found that using centricon ultrafiltration coupled with liquid scintillation counting was the most sensitive means for detecting covalently bound enzyme species containing ^{14}C.

A representative time course for a single-turnover experiment in which the conversion of [^{32}P]PEP to [^{32}P]P_i was examined is shown in Figure 3. The data showing the formation of product are fit to a single exponential to give a rate of 95 s^{-1}. This rate is consistent with that determined for the exponential phase in the pre-steady-state burst experiment.

A representative SDS−PAGE protein gel analysis of a reaction mixture in which we sought ^{32}P-labeled enzyme intermediates is shown in Figure 4. Three important pieces of information are provided by the experiments summarized in Table 1. First, there are *no* new radiolabeled peaks, which might correspond to an enzyme intermediate such as the cyclic bisphosphate, **2**, observed under single-enzyme turnover conditions. Second, the protein gel and centricon centrifugation analysis indicates that there are no covalent enzyme intermediates formed during catalysis. Finally, experiments in which we attempted to use radiolabeled products to examine the reverse reaction establish that the reaction is not reversible.

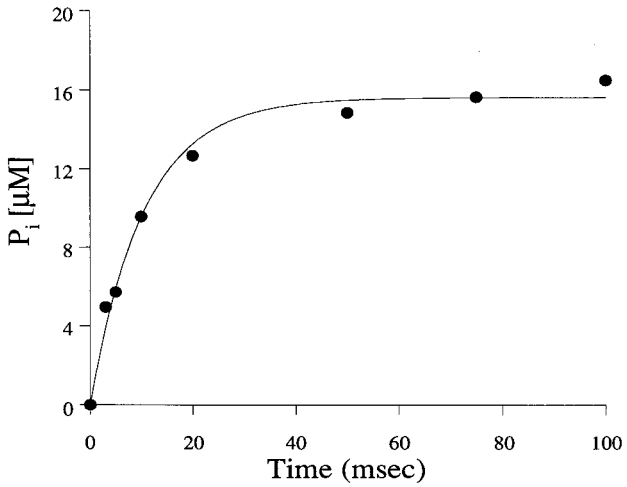


FIGURE 3: Kinetics of a single-turnover experiment in Kdo8P synthase. A solution containing enzyme (50 μM) preincubated with [^{32}P]PEP (20 μM) was mixed with A5P (250 μM) at 25 °C (final concentrations). The reaction was terminated at various time intervals by quenching with 0.2 N KOH, and the formation of product, P_i (●), was monitored by HPLC with radioactivity detection. The curve represents a fit of the data to a single exponential with a rate of 95 s^{-1}.

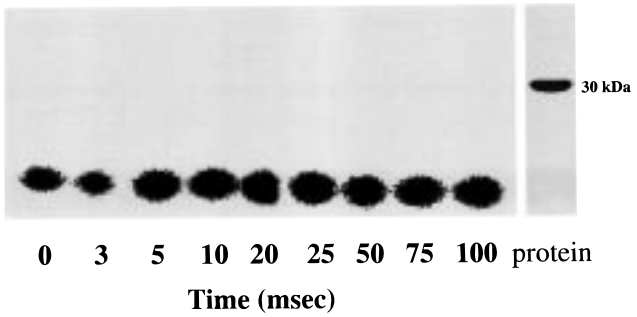


FIGURE 4: Gel analysis of a single-turnover experiment with Kdo8P synthase during which we looked for a covalent enzyme intermediate. Using the same conditions as described in Figure 3, aliquots of the quenched reaction mixture at different time intervals were loaded on SDS−PAGE and analyzed as described in Experimental Procedures. The polyacrylamide gel shows the radiolabeled components quenched at various time points during the single-turnover reaction. The protein lane shows a reference sample of Kdo8P synthase quenched under the same conditions as in the single-turnover experiments. No radiolabel is associated with the protein at any time point.

*Evaluation of Synthetic Cyclic Bisphosphate, **2**, as a Substrate for Kdo8P Synthase.* To further examine the possibility that the cyclic bisphosphate, **2**, may be an intermediate in the Kdo8P synthase reaction, we evaluated

the reaction of **2** with enzyme under single-enzyme turnover conditions. First, the stability of **2** to the quenching conditions used in the rapid chemical quench experiments was established by mixing with a solution of 0.2 N KOH followed by HPLC analysis. The retention time of a synthetic sample of **2** was determined by analyzing HPLC fractions by using a phosphate assay (*34*). The retention time was determined to be 40 min using the same HPLC conditions employed to separate substrates and products (see Experimental Procedures). No new radiolabeled HPLC peaks having a retention time of 40 min were observed in the single-turnover experiments described in Table 1. The fact that we established that the cyclic bisphosphate, **2**, was stable under our quenching conditions indicates that if **2** were formed in our single turnover experiments, we should have detected it by HPLC with radioactivity detection. However, in our rapid chemical quench experiments, we were not able to see any species beside substrate and product.

*Interaction of β-Kdo 2,8-Bisphosphate, **2**, with the Enzyme.* A synthetic sample of the cyclic bisphosphate, **2**, was further evaluated as a substrate of Kdo8P synthase. We expected that if **2** had been a true intermediate in the Kdo8P synthase-catalyzed reaction, then the enzyme should have been capable of catalyzing its decomposition by producing Kdo8P and inorganic phosphate. Since anomeric phosphate linkages are very sensitive under either thiobarbituric acid assay (*37*) or inorganic phosphate assay (*34*) conditions, we used a proton-decoupled ^{31}P NMR assay in which the appearance of inorganic phosphate could be clearly monitored. Representative NMR spectra obtained for the ^{31}P NMR time course over a 12 h period in the absence and presence of enzyme are shown in Figure 5.

Spectrum a in Figure 5 was taken on a standard solution of **2** (5 mM) at pD 8.5. Since we have found that **2** is unstable at neutral and especially at acidic pH, in which it undergoes rapid hydrolysis of anomeric phosphate, we prepared aliquots of a 10 mM solution in D_2O (pD 8.5) and stored them at −70 °C for preservation. Even under these conditions, this solution was not stable for extended periods of time, and the compound hydrolyzes slowly to produce Kdo8P and P_i. This hydrolysis can be seen in Figure 5a in which in addition to the resonances of two phosphates of **2** (8-P and 2-P), the decomposition of **2** to Kdo8P (8-P) and P_i is observed. The spectrum shown in Figure 5b represents the time course of decomposition of **2**, after incubation with Tris-DCl buffer (0.1 M) and bovine serum albumin (1.5 mg/mL) and adjustment of the pD to 7.0 in the absence of enzyme at 25 °C. The anomeric phosphate resonance of **2** (−1.7 ppm, 2-P) gradually diminishes in intensity with time and is replaced by a new singlet at δ (2.6 ppm) of inorganic phosphate, and by the 8-P resonances (4.6−5.4 ppm) of the anomeric mixture of the product Kdo8P.

To evaluate **2** as a substrate of Kdo8P synthase, we carried out several experiments under several different conditions. First, **2** was incubated [0.1 M Tris-DCl buffer and 1.5 mg/mL bovine serum albumin (pD 7.0)] with a 1000-fold higher concentration of the enzyme than is typically included in an enzyme assay experiment, and the reaction progress was monitored by ^{31}P NMR over a 24 h period (spectra not shown). No significant difference in the extent of anomeric phosphate release was detected versus the blank experiments (same conditions, but without enzyme), either at room temperature (25 °C) or at 37 °C. Further increases in the enzyme concentration (up to 8 mg of enzyme per experiment) yielded similar results; i.e., no enzyme-catalyzed acceleration in the anomeric phosphate hydrolysis was detected. The half-lives for the hydrolysis of **2** (pD 7.0) at 25 and 37 °C were estimated to be 4.7 and 2.5 h, respectively. These values are similar to those previously reported for β-Kdo 2-phosphate (*25*). The hydrolysis products of **2** were Kdo8P and P_i, as has been demonstrated in a separate experiment by following the reaction progress simultaneously in ^{31}P and ^{1}H NMR and through comparison of spectra for an authentic sample of Kdo8P.

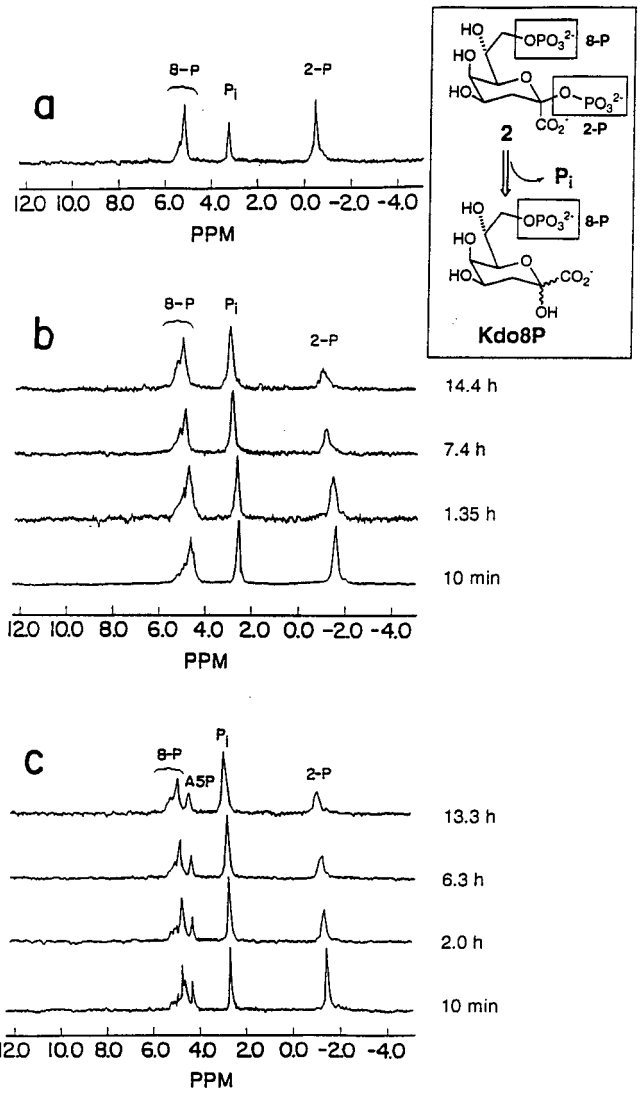


FIGURE 5: Time course of proton-decoupled ^{31}P NMR spectra showing conversion of **2** to Kdo8P and inorganic phosphate (P_i). Spectrum a was recorded on a standard solution of **2** (5 mM) at pD 8.5. (b) The time course of a control experiment with **2** (5 mM) and bovine serum albumin (1.5 mg/mL) in 0.1 M Tris-DCl buffer at pD 7.0 and 25 °C. (c) These spectra were obtained after certain time intervals from the addition of Kdo8P synthase (72 units, 8 mg) and A5P (1 mM) to the buffered solution of **2** (5 mM) at pD 7.0 and 25 °C. The anomeric phosphate (2-P) resonance of **2** at −1.7 ppm gradually diminishes with time and is replaced by the resonance of inorganic phosphate at 2.6 ppm. The resonances in the region of 4.6−5.4 ppm (8-P) correspond to the C-8 phosphates of **2** and of the anomeric mixture of the product Kdo8P.

Since we have shown that the enzyme is isolated with 1 equiv of PEP bound, it is possible that access to the active

Scheme 3: A Kinetic Reaction Pathway for Kdo8P Synthase

$$E + PEP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\bullet}PEP + A5P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\bullet}PEP{\bullet}A5P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \boxed{E{\bullet}I} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E{\bullet}P_i{\bullet}Kdo8P$$

$$E{\bullet}P_i{\bullet}Kdo8P \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} P_i + E{\bullet}Kdo8P \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} Kdo8P + E$$

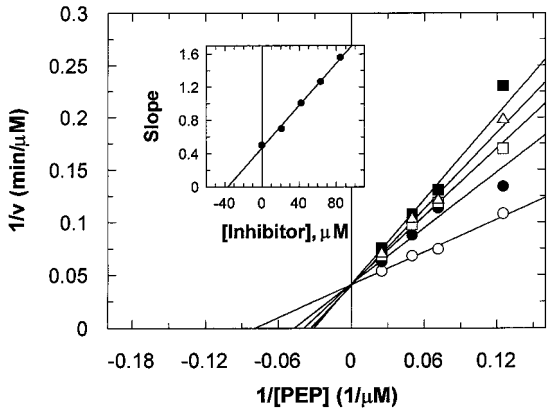


FIGURE 6: Inhibition of Kdo8P synthase by **2**. Double-reciprocal plots of initial velocities are given as a function of PEP concentration, where the A5P concentration is 500 μM and the inhibitor concentrations are 0 (○), 21 (●), 42 (□), 63 (■), and 84 μM (△). The assays were carried out at 37 °C and pH 7.3 (Tris-acetate buffer at 100 mM), including 38 milliunits of homogeneous Kdo8P synthase, PEP, A5P, and inhibitor (**2**) in a final volume of 1 mL. All samples were assayed in triplicate, and analogous results were obtained in two to four different experiments.

site by cyclic bisphosphate **2** may be partially blocked by PEP which thus may explain the failure of **2** to serve as a substrate of the synthase. To eliminate such a possible inhibitory effect of the enzyme with bound PEP, a similar set of experiments for the determination of the substrate activity of **2** were repeated with the presence of excess A5P (1 mM), which was directly introduced into the assay mixture (Figure 5c). This does not appear to be the case, however, as these experiments yielded results very similar to those obtained in the absence of A5P; i.e., compound **2** failed to show any detectable enzyme-catalyzed acceleration in the phosphate hydrolysis.

Having obtained these results, we then evaluated compound **2** as an inhibitor of the synthase. The kinetic behavior of **2** revealed that it is competitive with respect to PEP binding (Figure 6). The observed competitive inhibition versus PEP was anticipated on the basis of the kinetic mechanism (*22*). Since steady-state experiments have indicated that the enzyme adopts an ordered sequential kinetic pattern with PEP as a first substrate, it was anticipated that compound **2**, which combines the structural determinants of both substrates, would compete with PEP in the same, free enzyme form. The inhibition constant could be calculated from initial velocity studies and was estimated to be 35 μM.[2]

DISCUSSION

Kdo8P Synthase Kinetic Reaction Pathway. A transient kinetic analysis has provided a quantitative description of the Kdo8P synthase reaction pathway. Previous steady-state kinetic studies have suggested that the reaction pathway is ordered with PEP binding first followed by A5P (*22*). However, substrate trapping experiments conducted under single-enzyme turnover conditions have shown that either PEP or A5P can bind to enzyme in a kinetically competent mode to carry out chemical catalysis, suggesting a random mechanism. Therefore, the implications for the differences observed under steady-state and single-turnover conditions require further study. We have observed that enzyme is isolated with 1 equiv of PEP bound which may serve to enhance enzyme stability. Pre-steady-state burst experiments have shown biphasic formation of products (Kdo8P and P_i), indicating that chemical catalysis does not limit the overall reaction and the release of the product Kdo8P (k_6, Scheme 3), is at least partially rate-limiting. Since there is a faster step which governs catalysis, any enzyme reaction intermediates formed may transiently accumulate during a single enzyme turnover, thus enhancing the opportunity for detection. A key question in considering a minimal kinetic mechanism with an enzyme intermediate (the E·I complex, Scheme 3) is related to the relative rates of formation, k_3, and decomposition, k_4, of the intermediate and whether the intermediate may accumulate to a level sufficient to allow detection. Since we and others (*17*) have shown that the Kdo8P synthase reaction is not reversible, a related question is whether k_3, or k_4, is the irreversible step. Studies aimed at addressing these questions using analogues of the natural substrates are underway.

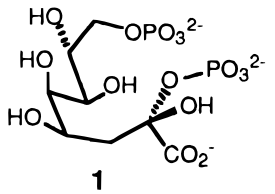
The True Enzyme Reaction Intermediate. The mechanism of catalysis, including enzyme reaction intermediates for Kdo8P synthase, has been the subject of considerable controversy. Plausible suggestions have been proposed for intermediates in the enzyme-catalyzed reaction, but direct experimental evidence for distinguishing among alternative pathways is lacking. The suggestion of the cyclic bisphosphate, **2**, as a putative reaction intermediate in the Kdo8P synthase-catalyzed reaction was based on reasonable chemical grounds and on the results accumulated through the synthesis and examination of various analogues of the substrates, product, and possible intermediate mimics. However, these observations only provide *indirect* evidence that the cyclic bisphosphate, **2**, may be a reaction intermediate.

Our present studies were designed to provide a means of *directly* establishing that the cyclic bisphosphate is indeed a true reaction intermediate using parallel transient kinetic and synthetic approaches. Collectively, our data from both kinetic and synthetic approaches show that the cyclic bisphosphate, **2**, is neither a reaction intermediate nor a substrate for Kdo8P synthase but rather a modest inhibitor of the enzyme. Therefore, we conclude that the cyclic bisphosphate, **2**, is not an intermediate in the normal reaction pathway catalyzed by the enzyme (as illustrated in Scheme 2, path b). This conclusion is based upon several lines of evidence.

[2] Due to a typesetting error, the K_i was previously reported (page 2467 of ref *29*) to be 35 mM. The correct value for the K_i is 35 μM.

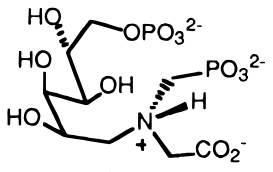
First, rapid chemical quench experiments in which radiolabeled substrates were used (either ^{14}C- or ^{32}P-labeled A5P or PEP) conducted under single-enzyme turnover conditions and basic quenching conditions to terminate the enzymatic reaction did not detect the formation of intermediate structures such as **2**. In addition, further analysis of the reaction mixtures by centricon and SDS-PAGE protein gel analysis failed to detect the formation of any covalently bound enzyme intermediates. Since the cyclic bisphosphate **2** is very stable under basic conditions (*25*), we would have detected **2** as a new radiolabeled peak following HPLC analysis of single-turnover reactions. The HPLC retention time was established using a synthetic sample of **2**. Second, ^{31}P NMR studies in which the rate of hydrolysis of **2** to Kdo8P and P_i was examined showed no rate enhancement in the presence of enzyme. Additionally, enzyme inhibition assays have shown that **2** is a competitive inhibitor with a K_i of 35 μM.

Having ruled out the cyclic bisphosphate, **2**, as a reaction intermediate as well as any stable covalent enzyme intermediates, we are left with the question of the identity of the true reaction intermediate. Taken together, the available data point to a mechanism that involves the formation of the acyclic hemiketal bisphosphate **1** as a reaction intermediate, originally proposed (*17*) and illustrated in Scheme 2 (path a). If the reaction intermediate is indeed the acyclic bisphosphate intermediate, **1**, we might predict that conformational flexibility may be a key component in the process of recognition at the enzyme active site. The moderate inhibitory activity ($K_i = 35$ μM) of the cyclic bisphosphate, **2**, may be due, in part, to a poor fit in which restricted compact cyclic structures, such as **2**, do not fit well within the flexible spatial orientation imposed by **1** onto the enzyme binding site. These results appear to be consistent with the proposal that structure **1** is the correct intermediate structure utilized by the enzyme during catalysis. In addition, very recently (*38*), we have synthesized the first bisubstrate inhibitor of the enzyme that combines the key features of A5P and PEP into a single molecule and has an acyclic structure (structure **3**). This compound has been shown to be the most potent inhibitor of the enzyme with a K_i value of 3.3 μM. This observation, coupled with these results, provides additional support for the concept of a mechanism that proceeds through the involvement of an acyclic bisphosphate intermediate **1**.



1

Proposed acyclic bisphosphate intermediate



3

Acyclic inhibitor

A definitive confirmation of the acyclic bisphosphate **1** as a true reaction intermediate can only be established by observation and characterization of this chemical species during enzyme turnover or by synthesizing **1** and showing that it can be converted to products (Kdo8P and P_i) on a time scale relevant to enzyme catalysis. The inherent chemical lability of **1**, as a hemiketal phosphate, makes direct observation and/or synthesis very challenging. For instance, rapid chemical quench methods designed to isolate and characterize the acyclic bisphosphate would likely result in its decomposition. We have recently developed a new methodology termed rapid mixing, pulsed-flow ESI with which chemically labile enzyme intermediates can be detected without the need for chemical quenching (*11*). Studies in which the rapid mixing, pulsed-flow ESI technique is used to examine the Kdo8P synthase reaction under single-enzyme turnover conditions in an effort to examine labile enzyme intermediates are underway. A parallel synthetic effort to synthesize **1** and characterize its interaction with Kdo8P synthase is also ongoing.

ACKNOWLEDGMENT

We thank Dr. Valery Belakhov and Shoucheng Du for help and advice. We gratefully acknowledge Dr. D. Dunaway-Mariano at the University of New Mexico (Albuquerque, NM) for supplying us with the generous gift of pyruvate phosphate dikinase.

SUPPORTING INFORMATION AVAILABLE

Complete details for the synthesis and spectral characterization of the cyclic bisphosphate **2** (7 pages). Ordering information is given on any current masthead page.

REFERENCES

1. Walsh, C. T., Benson, T. E., Kim, D. H., and Lees, W. J. (1996) *Curr. Biol. 3*, 1074.
2. Haslam, E. (1993) in *Shikimic Acid. Metabolism and Metabolites*, Wiley, New York.
3. Kahan, F. M., and Kahan, J. S. (1974) *Ann. N.Y. Acad. Sci. 235*, 364.
4. Steinrucken, H. C., and Amrhein, N. (1980) *Biochem. Biophys. Res. Commun. 94*, 1207.
5. Wanke, C., and Amrhein, N. (1993) *Eur. J. Biochem. 218*, 861.
6. Steinrucken, H. C., and Amrhein, N. (1984) *Eur. J. Biochem. 143*, 351.
7. Anderson, K., Sikorski, J., and Johnson, K. (1988) *Biochemistry 27*, 1604−1610.
8. Anderson, K. S., Sikorski, J. A., and Johnson, K. A. (1988) *Biochemistry 27*, 7395−7405.
9. Anderson, K. S., and Johnson, K. A. (1990) *Chem. Rev. 90*, 1131−1149.
10. Anderson, K., and Johnson, K. (1990) *J. Biol. Chem. 265*, 5567−5572.
11. Paiva, A., Tilton, R. F., Crooks, G. P., Huang, L. Q., and Anderson, K. S. (1997) *Biochemistry 36*, 15472−15476.
12. Studelska, D., McDowell, L., Espe, M., Klug, C., and Schaefer, J. (1997) *Biochemistry 36*, 15555−15560.
13. Marquardt, J., Brown, E., Walsh, C. T., and Anderson, K. S. (1993) *J. Am. Chem. Soc. 115*, 10398−10399.
14. Brown, E., Marquardt, J., Lee, J., Walsh, C., and Anderson, K. (1994) *Biochemistry 33*, 10638−10645.
15. Anderson, L., and Unger, F. M., Eds. (1983) *Bacterial Lipopolysaccharides*, ACS Symposium Series 231, American Chemical Society, Washington, DC.
16. Inouye, M. (1979) in *Bacterial Outer Membrane: Biogenesis and Function*, Wiley, New York.
17. Hedstrom, L., and Abeles, R. (1988) *Biochem. Biophys. Res. Commun. 157*, 816−820.
18. Dotson, G. D., Nanjappan, P., Reily, M. D., and Woodard, R. W. (1993) *Biochemistry 32*, 12392−12397.
19. Dotson, G. D., Dua, R., Clemens, J., Wooten, E. M. D., and Woodard, R. W. (1995) *J. Biol. Chem. 270*, 13698.
20. Bassov, T., Sheffer-Dee-Noor, S., Kohen, A., Jakob, A., and Belakhov, V. (1993) *Eur. J. Biochem. 217*, 991−999.

21. Woisetschlager, M., and Hogenauer, G. (1986) *J. Bacteriol. 168*, 437−439.
22. Kohen, A., Jakob, A., and Baasov, T. (1992) *Eur. J. Biochem. 208*, 443.
23. Kohen, A., Berkovich, R., Belakhov, V., and Baasov, T. (1993) *Bioorg. Med. Chem. Lett. 3*, 1577.
24. Ray, P. H., Kelsey, J. E., Bigham, E. C., Benedict, C. D., and Miller, T. A. (1983) in *Bacterial Lipopolysaccharides* (Anderson, L., and Unger, F. M., Eds.) pp 141−170, ACS Symposium Series 231, American Chemical Society, Washington, DC.
25. Bassov, T., and Kohen, A. (1995) *J. Am. Chem. Soc. 117*, 6165−6174.
26. Benenson, Y., Belakhov, V., and Baasov, T. (1996) *Bioorg. Med. Chem. Lett. 6*, 2901−2906.
27. Sheffer-Dee-Noor, S., Belakhov, V., and Bassov, T. (1993) *Bioorg. Med. Chem. Lett. 3*, 1583−1588.
28. D'Souza, F. W., Benenson, Y., and Baasov, T. (1997) *Bioorg. Med. Chem. Lett. 7*, 2457−2462.
29. Liang, P. H., Kohen, A., Baasov, T., and Anderson, K. S. (1997) *Bioorg. Med. Chem. Lett. 7*, 2463−2468.
30. Bednarski, M. D., Crans, D. C., DiCosimo, R., Simon, E. S., Stein, P. D., and Whitesides, G. M. (1988) *Tetrahedron Lett. 29*, 427−430.
31. Hirschbein, B. L., Mazenod, F. P., and Whitesides, G. M. (1982) *J. Org. Chem. 47*, 3765−3766.
32. Johnson, K. A. (1992) *Enzymes 20*, 1−61.
33. Schoner, R., and Herrmann, K. M. (1976) *J. Biol. Chem. 251*, 5440−5447.
34. Ames, B. N. (1966) *Methods Enzymol. 8*, 115−118.
35. Leatherbarrow, R. J. (1990) *GraFit*, version 2.0, Erithacus Software Ltd., Staines, U.K.
36. Segel, I. H. (1976) *Biochemical Calculations*, 2nd ed., Wiley, New York.
37. Ray, P. (1980) *J. Bacteriol. 141*, 635−644.
38. Du, S., Tsipori, H., and Baasov, T. (1997) *Bioorg. Med. Chem. Lett. 7*, 2469−2472.

BI981374W