Communication

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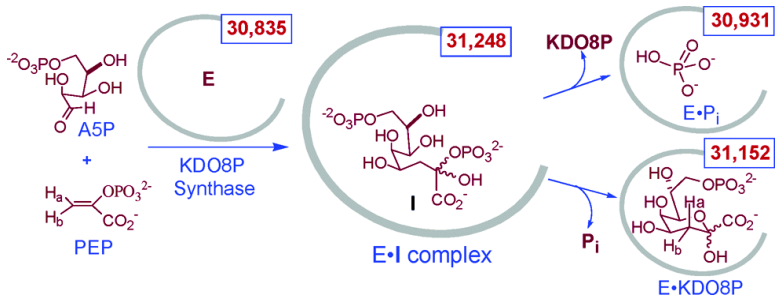

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A Snapshot of Enzyme Catalysis Using Electrospray Ionization Mass Spectrometry

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In this communication we report the first direct identification of the inherently unstable putative hemiketal phosphate intermediate bound to the enzyme 3-deoxy-D-*manno*-2-octulosonate-8-phosphate synthase (KDO8PS) in a noncovalent complex. This is accomplished by the application of time-resolved electrospray ionization mass spectrometry (ESI-MS) experiments directly monitoring the enzymatic reaction with its natural substrates on a very short, millisecond time scale.

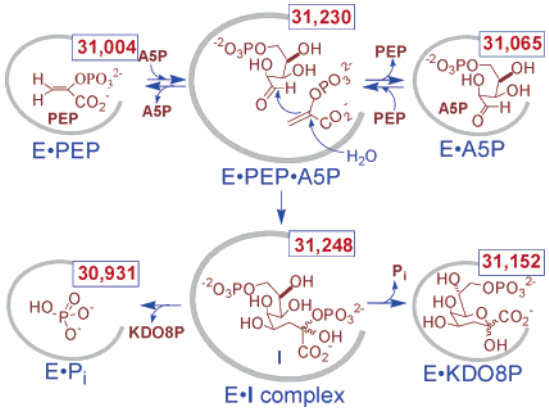
KDO8PS catalyzes the first committed step in the lipopolysaccharide biosynthetic pathway in Gram negative bacteria and involves a net aldol condensation between arabinose-5-phosphate (A5P), H_2O, and phosphoenol pyruvate (PEP) to form an unusual eight-carbon sugar KDO8P and inorganic phosphate (P_i) (Scheme 1).[1] Since KDO8PS is essential for Gram negative bacteria and not present in mammalian systems, it represents an attractive molecular target for the design of new antibiotics.[2]

A number of biochemical[3] and structural[4] studies suggest that the reaction mechanism of KDO8PS may involve the transient formation of a labile hemiketal phosphate enzyme intermediate (**I**, Scheme 1). In addition, we have recently synthesized the first bisubstrate inhibitor combining the key features of PEP and A5P into a single molecule which has an acyclic structure.[5] This compound proved to be potent inhibitor of KDO8PS with a K_d of 400 nM, lending support for the concept of a mechanism that proceeds through the involvement of intermediate **I**. However, despite all of the above observations, to date there is no evidence available for the existence of **I** as a true enzymatic intermediate. Moreover, this type of hemiketal phosphate species, while inferred in several chemical and enzymatic reactions,[6] has not yet been directly observed. This is due to the fact that the proposed hemiketal phosphate is both chemically labile and likely to have a very short half-life, whether free in solution or bound to the enzyme.[3b,6]

Previous investigations have demonstrated the successful application of ESI-MS and matrix-assisted laser desorption/ionization (MALDI) to monitor enzymatic reactions on longer time scales (>0.1 s) and to detect low-molecular weight enzyme intermediates as well as covalently bound enzyme intermediates.[7,8] The current study focused on the simultaneous detection of substrates and products as well as the putative intermediate **I** that are bound to the enzyme as *noncovalent* complexes, to establish the catalytic mechanism for KDO8PS.

A transient kinetic approach has been successfully used for direct detection of both noncovalent and covalent enzyme intermediates[9] on a millisecond time scale with the utilization of rapid chemical quench methodology.[10] However, a limitation of this methodology is the detection of intermediates that are chemically labile under quench conditions, e.g. acidic or basic, and hence precludes direct detection. This is, in fact, the presumed problem for detection of the labile hemiketal phosphate intermediate (**I**) hypothesized for KDO8PS. Also, while our previous transient kinetic studies of KDO8PS indicated that the half-life for this reaction is ~7 ms,[3b] the reaction is completely irreversible[3a,b] precluding the use of equilibrium or reverse direction experiments to examine catalysis. Considering the above limitations, our strategy for the detection of **I** involved the design of a novel rapid-mixing technique interfaced with high-resolution ESI-MS that would allow real-time monitoring of chemical catalysis for enzyme reaction times as short as 6−7 ms, hence avoiding the need for chemical quenching. To achieve the required short time resolution, the ESI time-of-flight (ESI-TOF) mass spectrometer has proven suitable for these studies.

Scheme 1. KDO8P synthase reaction pathway



Control experiments (see Supporting Information) were first conducted with the KDO8PS alone and with the binary complex KDO8PS·PEP (E·PEP) to ascertain ESI-TOF MS conditions for both optimal sensitivity and temporal resolution. The desired short-time resolution was achieved by a combination of a custom-designed electrospray probe, high flow rates, and minimal length, narrow-bore fused silica capillary tubing. For maintaining efficient desolvation of the ions at the high flow rates used, high voltage (~120 V) was required that still allowed ~15−20% of noncovalent complexes to remain.

The catalytic reaction[11] of KDO8PS[12] with its natural substrates, PEP and A5P, was examined under single enzyme turnover conditions[13] by rapidly mixing the E·PEP solution with a limiting amount of the second substrate, A5P. The reaction was monitored over several time ranges (7 ms to 160 ms) as illustrated in Figure 1. At earlier times, the enzyme binary complexes with each substrate, E·PEP (*m*/*z* 2067.9) and E·A5P (*m*/*z* 2072.0), and each product, E·KDO8P (*m*/*z* 2077.8) and E·P_i (*m*/*z* 2063.1), along with the intense peak of the free enzyme (*m*/*z* 2056.7) are observed. Moreover, a resolved peak corresponding to the enzyme-bound intermediate (E·**I**, *m*/*z* 2084.2) is clearly evident (see Scheme 1

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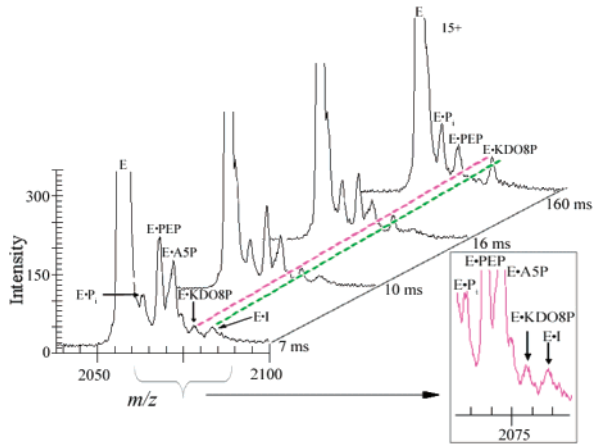

Figure 1. ESI-TOF mass spectra of KDO8PS during catalysis. A time course of KDO8PS catalysis during single turnover reaction monitored by ESI-TOF MS in the positive ion mode. The E·PEP was mixed with the second substrate, A5P. Each trace represents the average of 15 mass spectra recorded in trap pulse mode with 60 000 pulses per mass spectrum. The peaks marked E, E·P_i, E·PEP, E·A5P, E·KDO8P, and E·I correspond to KDO8PS and its complexes with P_i, PEP, A5P, KDO8P, and intermediate I, respectively, at 15+ charge state at the reaction times: 7, 10, 16, and 160 ms.

Table 1. Enzyme Complexes at Charge State 15+ and Corresponding Molecular Weights

complexes of enzyme catalysis and measured mass-to-charge ratio (*m*/*z*)		measured M_r (Da)	calculated M_r (Da)
$[E + 15H]^{15+}$	2056.7 ± 0.2	30835 ± 3	30834.5
$[E{\cdot}P_i + 15H]^{15+}$	2063.1 ± 0.1	30931 ± 2	30932.5
$[E{\cdot}PEP + 15H]^{15+}$	2067.9 ± 0.1	31004 ± 2	31002.5
$[E{\cdot}A5P + 15H]^{15+}$	2072.0 ± 0.1	31065 ± 2	31064.6
$[E{\cdot}KDO8P + 15H]^{15+}$	2077.8 ± 0.2	31152 ± 3	31152.7
$[E{\cdot}I + 15H]^{15+}$	2084.2 ± 0.1	31248 ± 2	31250.7

and Table 1 for structures and molecular weights) with similar intensity to the peak corresponding to E·KDO8P at the earliest time point. While we do not focus on quantitative kinetics of the reaction at this time, it is noted that the peaks corresponding to the products complexes [E·KDO8P (denoted by pink dotted line) and E·P_i] increase with longer reaction times, and the peaks corresponding to the substrates complexes (E·PEP and E·A5P) simultaneously decrease until there is complete consumption of the limiting substrate, A5P. Moreover, the peak corresponding to the E·**I** (denoted by the green dotted line), completely disappears in a time-dependent manner consistent with conversion of substrates to products as would be expected for a true reaction intermediate. Therefore, the observed molecular weight and kinetic characteristics of the E·**I** species establish that **I** is formed during the enzyme catalysis and has hemiketal phosphate structure as depicted in Scheme 1.[14]

In conclusion, this study establishes the catalytic mechanism for KDO8PS and describes the first *direct* evidence of a hemiketal phosphate intermediate, **I**, in enzyme catalysis. Further kinetic and ESI-TOF MS studies of the wild-type and mutant KDO8PS along with other enzymes involved similar hemiketal phosphate intermediate are underway. The rapid-mixing ESI-TOF MS has broad application for studying biological processes and opens up new routes to explore the earliest chemical events at the active site of an enzyme or receptor−ligand binding events.

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Supporting Information Available: More detailed information is provided for experimental rationale, design, interpretation, and controls (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Mass spectra were recorded by an Ettan ESI-TOF mass spectrometer (Amersham Biosciences) at skimmer voltage 120 V, analyzer pressure 2.2×10^{-7} Pa. A solution of 40 μM KDO8PS (in 10 mM NH_4OAc buffer, pH 7.8) containing ~1 equiv of bound PEP (E·PEP) was combined in a mixing tee with the second substrate A5P (30 μM) in the same buffer using two 1000 μL syringes and a syringe pump (final concentrations). The reaction solution from the mixing tee was continually introduced into the mass spectrometer through an in-house built electrospray probe. The experiments were repeated four times to confirm the reproducibility of the assembly process. The mass analyses reported here are within a mass accuracy of 0.2 Da.
(12) The homogeneous KDO8PS was isolated from *E. coli* (pET9d) BL21-(DE3) as previously described.[3b] Recombinant protein is a homotetramer (Radaev, S.; Dastidar, P.; Patel, M.; Woodard, R. W.; Gatti, D. L. *Acta Crystallogr.* **2000**, *D56*, 516−519) containing 284 amino acids per subunit with a calculated MW of 30834.5 Da.
(13) Even though that the K_d values for the binary complexes of KDO8PS have not been determined (K_m^{PEP} = 6 μM, K_m^{A5P} = 25 μM, K_i^{Pi} = 10 mM, K_i^{KDO8P} = 0.6 mM: Kohen, A.; Jakob, A.; Baasov, T. *Eur. J. Biochem.* **1992**, *208*, 443−449), the examination of the reaction under single turnover conditions optimizes active-site enzyme−ligand bound forms to allow their detection under ESI-TOF MS conditions.
(14) Note that in single turnover experiments in the reverse direction, combining the enzyme with the two reaction products, KDO8P and P_i, under conditions similar to those described in Figure 1, revealed only the E·KDO8P and E·P_i complexes (data not shown). Since there was no ternary E·KDO8P·P (which would have the same mass as the E·I), this observation further proves that the peak attributed to E·I in Figure 1 most likely represents the enzyme intermediate complex as well as the earlier conclusion[3a,b] that catalytic reaction is completely irreversible.

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