

Isolation and Structural Elucidation of a Tetrahedral Intermediate in the UDP-*N*-acetylglucosamine Enolpyruvoyl Transferase Enzymatic Pathway

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UDP-*N*-acetylglucosamine enolpyruvoyl transferase, encoded by the *Escherichia coli* *murZ* gene,¹ catalyzes the transfer of an enolpyruvoyl moiety from phosphoenolpyruvate (PEP) to the 3-hydroxyl of UDP-*N*-acetylglucosamine (UDP-GlcNAc) with the elimination of inorganic phosphate. This reaction, shown in Scheme I, constitutes the first committed step in bacterial peptidoglycan biosynthesis and is the target of the antibiotic phosphomycin. Substantial structural and functional homology (18.3% identical) exists between MurZ and *E. coli* 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase (AroA), the only other enzyme known to catalyze an analogous enolpyruvoyl transfer.^{1–3} Previous studies have shown unambiguously that the EPSP synthase reaction pathway proceeds without an enzyme-linked intermediate via a tetrahedral phospholactyl adduct.^{4,5} In contrast, it has been proposed that catalysis by MurZ proceeds through either an enolpyruvoyl or phosphoenolpyruvoyl covalent enzyme intermediate.^{6,7} That two enzymes exhibiting substantial homology and catalyzing similar chemical transformations would utilize different reaction mechanisms prompted us to further investigate the mechanism of catalysis by MurZ.

In this report we describe the isolation and structural determination of a tetrahedral intermediate which is not covalently associated with MurZ. Rapid quench of the reaction under single turnover conditions followed by anion-exchange HPLC separation demonstrated that an intermediate was formed within 5 ms after substrates were mixed with the enzyme (data not shown). The time-dependent decay of the intermediate matched the appearance of product in both the forward and reverse reactions, demonstrating directly the kinetic competence of the presumed intermediate.

Radiolabeled substrates were used to probe the identity of the intermediate. Single-turnover reactions (10 ms) were conducted using the labeled substrates [¹⁴C]UDP-GlcNAc, [¹⁴C]PEP, and [³²P]PEP. Analysis of these reactions revealed that the intermediate contained enolpyruvoyl and phosphate moieties from PEP as well as radiolabeled UDP-GlcNAc (Table I). In addition, MurZ catalyzed the decomposition of isolated intermediate to PEP and UDP-GlcNAc-EP.

Labeled [¹³C] intermediate was generated at the active site of the enzyme from UDP-GlcNAc and [¹³C]-2-PEP. Following

Scheme I

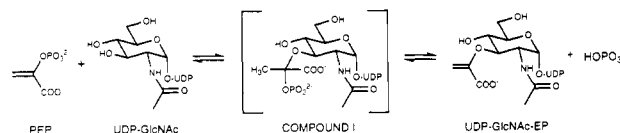


Table I. Synthesis of the Intermediate^a

radiolabel	[UDPG] (μM)	[PEP] (mM)	reactn time (ms)	% intermed	concn (μM)
[¹⁴ C]UDPG	25	5 mM	10	24	6.3
[¹⁴ C]PEP	100	25 μM	10	35	8.7
[³² P]PEP	100	25 μM	10	37	9.3

^a The indicated concentrations of substrates (Amersham), uridine diphospho-*N*-acetyl-D-[U-¹⁴C]glucosamine, phosphoenol[1-¹⁴C]pyruvate, and [1-³²P]phosphoenolpyruvate were incubated with 50 mM purified enzyme¹ for the specified time, and then the reaction was terminated by mixing with 0.2 N KOH (final concentrations). The percentage conversion of radiolabel into intermediate was quantitated by HPLC using a Mono-Q 5/5 anion-exchange column with a continuous flow radioactivity detector.^{4,9}

isolation under basic conditions, its structure was confirmed by ¹³C, ¹H, and ³¹P NMR. The intermediate was isolated from the enzyme reaction mixture as described in the legend to Figure 1. The ¹H NMR spectrum (Figure 1) was almost identical to that previously reported for UDP-*N*-acetylmuramic acid⁸ with the exception of the loss of the lactyl C-α proton at 4.32 ppm and the altered shift of three methyl protons at 1.36 ppm to 1.78 ppm. This distinctive methyl proton resonance was split into a doublet by the ¹³C at the tetrahedral center (see inset) with an apparent coupling constant of 3.9 Hz.

The proton-decoupled ¹³C NMR spectrum of the purified intermediate (Figure 1) revealed the presence of a resonance with a chemical shift of 104.0 ppm, suggestive of a tetrahedral carbon bearing two oxygens. This resonance was split into a doublet with a coupling constant of ²J_{CP} = 7.6 Hz (±0.4 Hz). Both the frequency and the coupling constant were similar to those observed for the tetrahedral intermediate of EPSP synthase (101.7 ppm and 7 Hz, respectively).⁵ The carbon-phosphorus coupling demonstrates that the phosphate is attached to the tetrahedral carbon in the intermediate. The carbon spectrum also demonstrated an extraneous peak at 163.1 ppm, which did not correspond to those for [¹³C]-2-PEP, [¹³C]-2-pyruvate, or UDP-GlcNAc-[¹³C]-2-EP acquired under the same conditions. Furthermore the resonance at 163.1 ppm was absent after repurification of the intermediate by anion-exchange HPLC (data not shown). A minor peak at 158.0 ppm was identified as the reaction product UDP-GlcNAc-[¹³C]-2-EP.

Three major phosphorus signals were observed for the intermediate as shown in the proton-decoupled ³¹P NMR spectrum in Figure 1. The two upfield signals correspond to the pyrophosphate linkage in the UDP moiety. A third signal at -5.7 ppm corresponds to the phosphate attached to the tetrahedral carbon of the intermediate and is analogous to that observed for the EPSP synthase intermediate (-4.6 ppm).⁵ A line width of 15 Hz obscures an anticipated coupling of 7.6 Hz with the ¹³C of the tetrahedral center.

The presence of the tetrahedral phospholactyl UDP-*N*-acetylglucosamine intermediate, as demonstrated above, suggests that MurZ utilizes a mechanism analogous to that of EPSP synthase. We therefore propose that the reaction catalyzed by MurZ proceeds via a direct attack of the 3-hydroxyl of UDP-

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(9) All enzyme reactions were performed at 25 °C in a buffer containing 50 mM TRIS at pH 8.0.

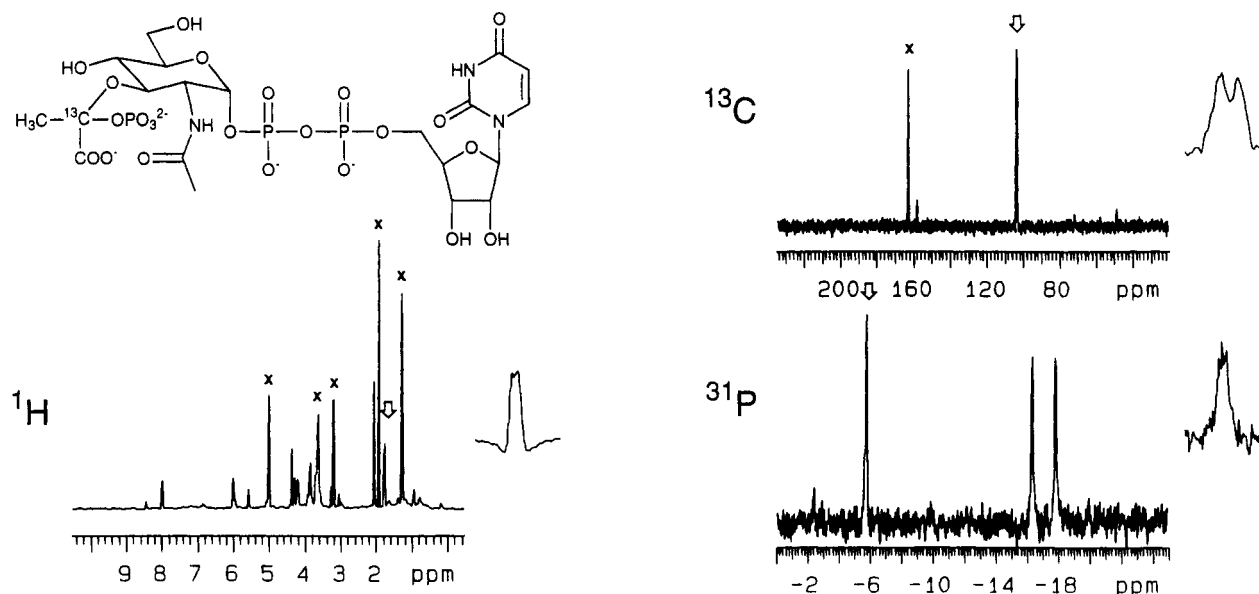


Figure 1. NMR spectra of the tetrahedral intermediate. Top inset: Structure of the intermediate. ^1H NMR spectrum: The proton spectra were obtained on a Varian VMX-500 at 500 MHz with 200 mM KOH in D_2O . Chemical shifts are in parts per million referenced to the H6'' of UDP-MurNAc at 8.00 ppm. The resonances for the protons are designated by the conventional numbering of the sugar ring (1–6), the ribose ring (1'–5'), and the uridine moiety (1''–6''). The unique methyl resonance is denoted by an arrow. Resonances denoted with an X were present in a sample containing only solvent. Inset: Expansion showing resolution-enhanced splitting of the methyl proton resonances (at 1.78 ppm) by the tetrahedral ^{13}C . ^{13}C NMR spectrum: The ^{13}C spectrum of purified intermediate was obtained on a Varian VMX-500 at 125.7 MHz with 200 mM KOH in D_2O . The spectrum was obtained with WALTZ ^1H decoupling and a 1.5-s delay between pulses. Dioxane was used as a reference ($\delta = 67.6$ ppm). The resonance designated by an arrow corresponds to that of the tetrahedral carbon, and that designated by an X was absent after repurification of the intermediate. Inset: Expansion showing resolution-enhanced splitting of the tetrahedral carbon (at 104.0 ppm) by phosphate. ^{31}P NMR spectrum: The phosphorus-31 spectrum of the intermediate was obtained on a Varian VMX-500 at 145.8 MHz in 200 mM KOH D_2O . Chemical shifts are reported in parts per million relative to potassium phosphate in 200 mM KOH. The spectrum was obtained with WALTZ ^1H decoupling and a 3-s delay between pulses. The resonance of the phosphate attached to the tetrahedral carbon is designated by an arrow. Inset: Expansion showing the phosphate (at -4.6 ppm) attached to tetrahedral carbon. Methods: Intermediate (600 μg) was synthesized enzymatically by mixing [^{13}C]-2-PEP (2 mM) with an equal volume (2 mL) or purified enzyme¹ (1 mM) and UDP-GlcNAc (2 mM). The reaction was allowed to proceed for 50 ms and quenched with KOH (final concentration 0.2 M). A trace amount of [^{14}C]PEP was included in the incubation to aid isolation of compound I. Denatured protein was removed from the quenched reaction mixture by membrane filtration, and the intermediate was isolated by chromatography on a Mono-Q 5/5 (LKB/Pharmacia) anion-exchange column (20–500 mM triethylammonium bicarbonate gradient). Samples were lyophilized and then resuspended in 0.2 M KOH in D_2O .

N-acetylglucosamine on the C-2 position of phosphoenolpyruvate to give the tetrahedral intermediate (compound I, Scheme I). Previous studies on MurZ have suggested an enolpyruvoyl or phosphoenolpyruvoyl intermediate based largely on the incorporation of small amounts of radioactive [^{14}C]PEP or [^{32}P]PEP in the enzyme after precipitation with ammonium sulfate or after

treatment with SDS.^{6,7} The isolation and structural elucidation in addition to our rapid quench kinetics provide definitive identification of the tetrahedral intermediate as a true intermediate in the UDP-GlcNAc enolpyruvoyl transferase reaction pathway. Studies to provide a complete kinetic and thermodynamic description of the enzyme reaction pathway are now in progress.