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CONVERSION OF THE COVALENT INTERMEDIATE 3-FLUORO-2-PHOSPHO-LACTYL-EPTase TO 3-FLUORO-2-PHOSPHOLACTYL-UDP-GLCNAC

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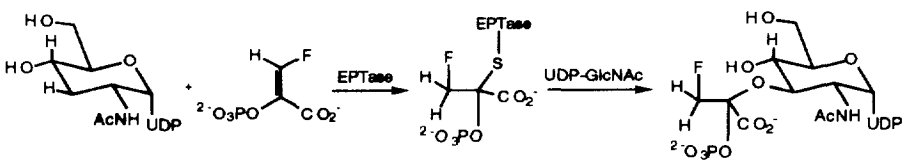
ABSTRACT: The 3-fluoro-2-phospholactyl-UDP-GlcNAc enolpyruvyl transferase (EPTase) intermediate is prepared by the incubation of 3-fluorophosphoenolpyruvate (3-FPEP) and 3-deoxy-UDP-GlcNAc with "PEP-free" UDP-GlcNAc enolpyruvyl transferase (EPTase). This intermediate is converted directly into the 3-fluoro-2-phospholactyl-UDP-GlcNAc intermediate by incubation with UDP-GlcNAc. Utilization of UDP-GalNAc as substrate results in the formation of two fluoro-containing tetrahedral intermediates but in a ratio different from that seen with UDP-GlcNAc.

Uridine diphospho-*N*-acetylglucosamine enolpyruvyl (UDP-GlcNAc-EP) transferase [EC 2.5.1.7] (EPTase) is the first unique enzyme in the biosynthesis of the muramic acid moiety of the peptidoglycan portion of the cell wall. UDP-muramic acid, the activated form of muramic acid, is formed by reduction of UDP-GlcNAc-EP by the second enzyme in the pathway. The successive addition of various amino acids to the lactoyl function of muramic acid leads to the polypeptide side chain that is ultimately crossed-linked in the outer cell wall. These cross-linked polypeptide side chains provide the structural rigidity for the microorganism's outer cell wall necessary for survival in hypoosmotic environments normally encountered by the microorganism. Interference with steps late in peptidoglycan biosynthesis has provided the basis for many of the present day antibiotics. However, emerging resistance to available antibiotics has provided the impetus to explore alternate enzymes in the peptidoglycan biosynthetic pathway, such as EPTase, as targets for the development of new antibiotics.

EPTase catalyzes the formation of UDP-GlcNAc-EP from UDP-*N*-acetylglucosamine (UDP-GlcNAc) and phosphoenolpyruvate (PEP).[1-5] To date, this unusual transfer of the carboxyvinyl moiety from PEP to another substrate with the elimination of inorganic phosphate is seen in only one other PEP-utilizing enzyme, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, the target enzyme of the commercial herbicide glyphosate [N-(phosphonomethyl)glycine].[6] The sequence homology between EPSP synthase and EPTase is significant (20% identity).[7,8] Anderson, et al.[6-9] have isolated and elucidated the structure of a single non-covalently-bound tetrahedral intermediate involved in the formation of EPSP by the combined use of rapid chemical quench techniques coupled with ^{1}H and ^{13}C-NMR. Evans, et al.[10] utilizing time-resolved solid-state NMR spectroscopy has also detected this transient enzyme intermediate complex. Alternate substrate studies of EPSP synthase revealed that (*Z*)-3-FPEP but not (*E*)-3-FPEP acted as a pseudosubstrate.[11] The (*Z*)-3-FPEP was converted into a tightly bound fluoro-containing analog of the tetrahedral intermediate which did not collapse to product.[11] It was originally reported that the reaction pathway used by EPTase, unlike EPSP synthase, involved an enzyme-bound intermediate.[5,12] Recently in a series of elegant experiments, Wanke and Amrhein[13] demonstrated in *Enterobacter cloacae* the involvement of a covalent-enzyme intermediate in which cysteine 115 was attached to the C-2 of PEP to form what they assumed to be an O-phosphothioketal intermediate. Walsh, et al.[14] reported that the mechanism of EPTase, like EPSP synthase, involved only the formation of a tetrahedral intermediate. Later, Walsh, et al.[15] revised their initial mechanism to involve the formation of both a covalently-bound enzyme intermediate as well as a non-covalently-bound tetrahedral intermediate. Based on

the results from the studies involving the incubation of 3-FPEP with EPSP synthase, we decided to incubate EPTase with 3-FPEP and monitor the reaction via ^{19}F-NMR to observe the formation of both tetrahedral intermediates. Prior to the completion of our studies, Walsh, et al.[16] published the results from their investigations involving incubation of 3-FPEP with MurZ (EPTase). The results from the Walsh laboratory and the results presented in this paper demonstrate the formation of a fluoromethyl-containing non-covalently-bound tetrahedral intermediate as well as a fluoro-containing covalently-bound tetrahedral intermediate. Unlike EPSP synthase, EPTase utilizes both stereoisomers of 3-FPEP.[16] It has been demonstrated that incubation of the fluoro-containing non-covalently-bound tetrahedral intermediate with "PEP-free" EPTase leads to the formation of a mixture of both tetrahedral intermediates equivalent to that formed by incubation of 3-FPEP with UDP-GlcNAc in the presence of EPTase. In this report, we, too, describe the formation of both fluoro tetrahedral intermediates by reaction of UDP-GlcNAc with 3-FPEP in the presence of "PEP-free" EPTase. More importantly, we report the direct formation of only the 3-fluoro-2-phospholactyl-UDP-GlcNAc enolpyruvyl transferase intermediate, as observed by ^{19}F-NMR, by incubation of 3-FPEP with "PEP-free" EPTase in the presence of **3-deoxy**-UDP-GlcNAc. Upon incubation with UDP-GlcNAc, the 3-fluoro-2-phospholactyl-UDP-GlcNAc-EPTase intermediate is converted into the 3-fluoro-2-phospho–lactyl-UDP-GlcNAc intermediate and EPTase. The ^{19}F-NMR spectrum of an incubation of "PEP-loaded" EPTase with 3-FPEP in the presense of **3-deoxy**-UDP-GlcNAc gave no observable ^{19}F-NMR signal other than that of the starting 3-FPEPs.



Scheme 1.

The EPTase was isolated from an overproducing strain, *E. coli* BL21 (DE3), containing the pT7-7-based plasmid pEPT that contains the murZ gene. Purification to homogeneity was accomplished by two sequential anion exchange columns (Fast flow Q, followed by Mono Q HR 10/10 (Pharmacia)). The dye-affinity chromatography methodology reported by Amrhein for EPTase from *E. cloacae* was to determine the state of substitution of the sulfur atom of cysteine 115[17] at the active site of EPTase. Wanke and Amrhein were able to separate pure EPTase into two different enzyme forms, "PEP-loaded EPTase (85%)" and "PEP-free EPTase (15%)", via chromatography on Reactive Yellow YA-86 (Sigma). The peak corresponding to "PEP-loaded EPTase" disappeared with the concomitant increase in the peak due to "PEP-free EPTase" along with the appearance of a peak corresponding to UDP-GlcNAc-EP upon treatment with an excess of the other substrate, UDP-GlcNAc. For the preparation of "PEP-free EPTase" used in this study, the pooled Mono Q enzyme was dialyzed against buffer B (25 mM Bis-tris propane/HCl pH 6.6, 2 mM DTT) with one buffer change. To cleanse the EPTase from PEP, the dialysate was incubated at 22° C for 10 min in the presence of 1mM UDPGlcNAc before loading onto a Reactive Yellow YA-86 that was previously equilibrated with buffer B. After an initial elution with buffer B, the bound EPTase was eluted with a linear gradient 0 - 0.4 M KCl in buffer B. The most active fractions were pooled and concentrated and the buffer was changed to buffer A by centrifugation (Microsep 10K, Filtron). The cleansed EPTase was stored at -80° C until used. The electrospray ionization mass spectra (ES-MS) of the "PEP-loaded EPTase" is identical to that of the "PEP-free EPTase".

Incubation of 3-FPEP and UDP-GlcNAc with "PEP-loaded EPTase" resulted in the formation of two fluoro-containing tetrahedral intermediates that can be observed by ^{19}F-NMR (Figure 1a.) as two broad singlets. The ^{19}F-NMR spectrum of this incubation mixture after treatment with KOH consists of a broad singlet and a sharp triplet (δ = -225 ppm) suggesting that one of the two tetrahedral intermediates was enzyme-bound (Figure 1b). These results were confirmed by comparing the ES-MS of "PEP-free EPTase" versus "PEP-free EPTase" incubated with 3-FPEP and UDP-GlcNAC (data not shown) and are in keeping with the results previously reported.[16] Incubation of 3-FPEP and the unreactive substrate analog 3-deoxy-UDP-GlcNAc in the presence of "PEP-loaded" EPTase resulted in the appearance of NO new resonances in the ^{19}F-NMR (spectra not shown). However, when the same incubation was repeated but in the presence of "PEP-free EPTase" a single new broad signal appeared in the ^{19}F-NMR spectrum (Figure 2a) which remained as a broad singlet after treatment with KOH (spectra not shown). These results suggest the exclusive formation of only the covalent 3-fluoro-2-phospholactyl-UDP-GlcNAc enolpyruvyl transferase intermediate. In an identical experiment, 3-FPEP and 3-deoxy-UDP-GlcNAc were incubated with "PEP-free EPTase". Following the removal of the (Z)-3-FPEP and 3-deoxy-UDP-GlcNAc from the reaction mixture by repeated ultrafiltration (Centricon 3)/buffer dilution, the addition of the natural cosubstrate UDP-GlcNAc resulted in the formation of predominantly one new tetrahedral intermediate as observed by ^{19}F-NMR (Figure 2b). This latter tetrahedral intermediate appears to be non-covalently bound to the enzyme since treatment of the incubation mixture with KOH produces a well-resolved triplet centered at -225 ppm as was observed in the original experiment (Figure 2c). These results, while clearly demonstrating that the enzyme-bound intermediate may be converted into the free tetrahedral intermediate, in no way imply that the formation of the 3-fluoro-2-phospholactyl-UDP-GlcNAc enolpyruvyl transferase intermediate must precede the formation of the 3-fluoro-2-phospholactyl-UDP-GlcNAc intermediate. Kinetic data concerning the inactivation of EPTase by Z-3-FPEP have recently been presented that suggest the mechanism of normal MurZ (EPTase) is branching.[18] While it has been demonstrated by ^{19}F-NMR studies[16] that incubation of the 3-fluoro-2-phospholactyl-UDP-GlcNAc intermediate with "PEP-free EPTase" leads to the formation of 3-fluoro-2-phospholactyl-UDP-GlcNAc enolpyruvyl transferase, these experiments represent the first ^{19}F-NMR experiments demonstrating the reverse reaction of the equilibrium. Evidence has been presented that suggests that the interconversion between these two fluoro-containing tetrahedral intermediates involve a common oxocarbenium ion rather than the direct displacement at the C-2 of the PEP moiety.

In another experiment, 3-FPEP and UDP-GalNAc (Sigma Chemical Co.) were incubated with "PEP-loaded EPTase". Inspection of the ^{19}F-NMR spectra of the incubation mixture before (Figure 1c) and after addition of KOH (Figure 1d) reveals the formation of both 3-fluoro-2-phospholactyl intermediates albeit in different ratios from that formed with the normal substrate, UDP-GlcNAc. The increase in the amount of enzyme-bound fluoro intermediate versus free tetrahedral intermediate suggests that the UDP-GalNAc is a poorer substrate than UDP-GlcNAc. This is not surprising since the conformational differences in the two pyranoses (the C-4-OH in galactosamine is axial versus equatorial in glucosamine) occur at the carbon atom adjacent to the reactive C-3-OH group. This altered conformation of the substrate allows the somewhat less favored reaction, namely C115 attack onto the C-2 of PEP, to become the major reaction pathway. Further mechanistic studies of EPTase involving UDP-GalNAc as an alternate substrate are presently ongoing.[19]

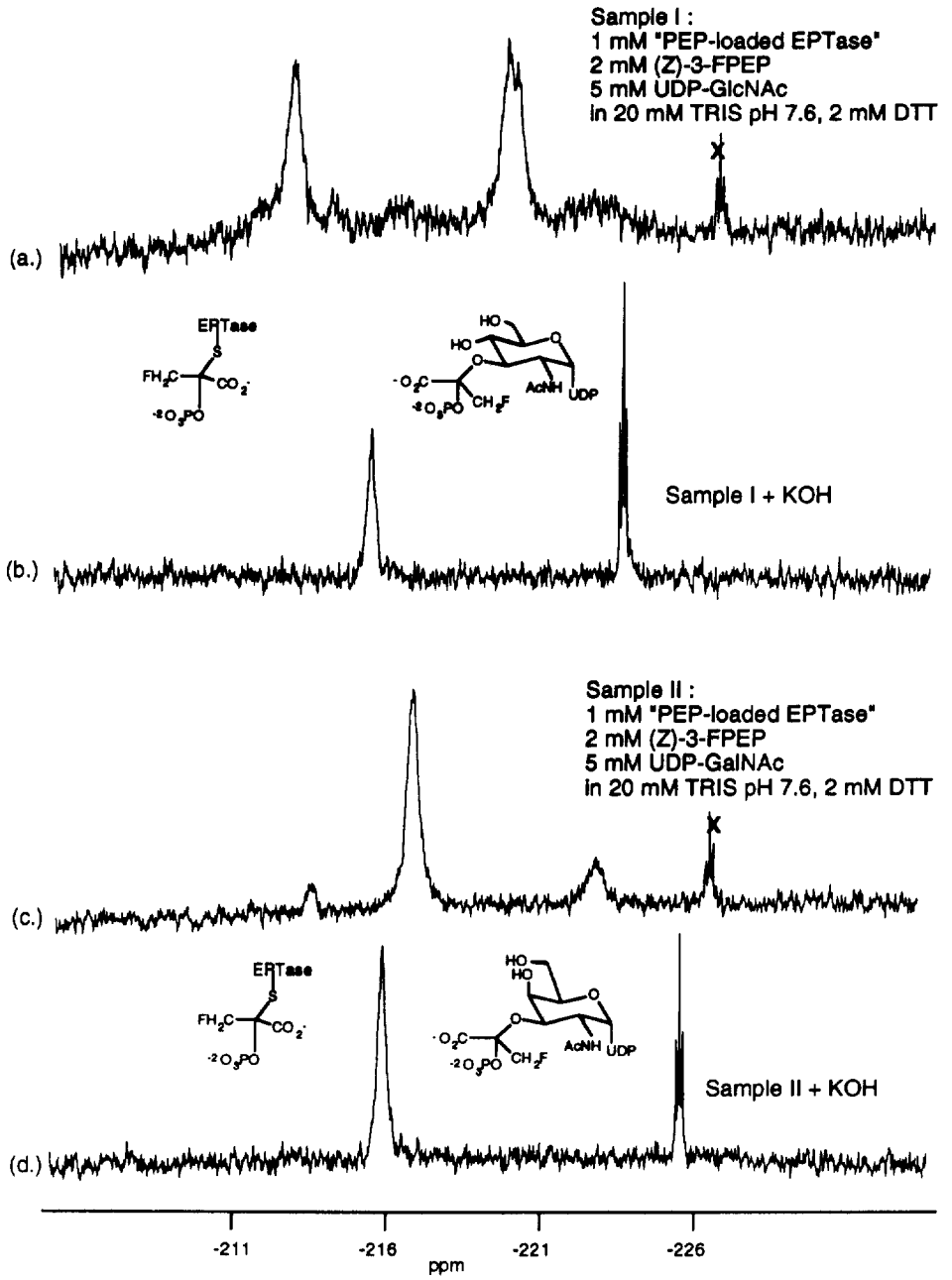


Figure 1: (a) ^{19}F-NMR spectra of "PEP-loaded EPTase" (1 mM) in the presence of (Z)-3-FPEP (2 mM) and UDP-GlcNAc (5 mM) in 20 mM Tris/HCl pH 7.6 containing 2 mM DTT, (b) after the addition of 0.2M KOH, (c) ^{19}F-NMR spectra of "PEP-loaded EPTase" (1 mM) in the presence of (Z)-3-FPEP (2 mM) and UDP-GalNAc (5 mM) in 20 mM Tris/HCl pH 7.6 containing 2 mM DTT,[19] (d) after the addition of 0.2M KOH. X peak is impurity in the 3-FPEP starting material. ^{19}F-NMR spectra were acquired on a GE 500 MHz operating at 470 MHz and are referenced to external Freon-11.

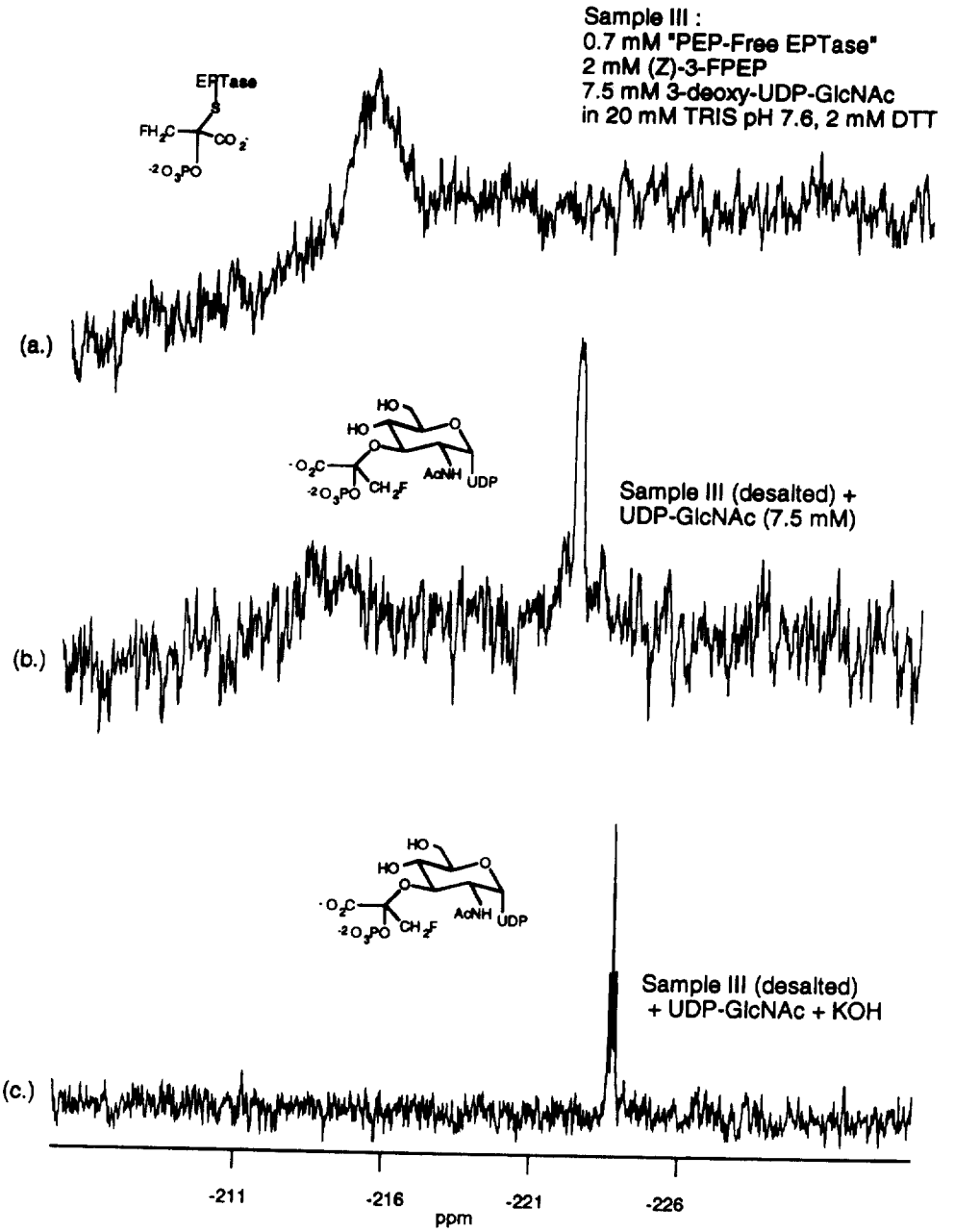


Figure 2: (a) ^{19}F-NMR spectra of "PEP-free EPTase" (0.7 mM) in the presence of (Z)-3-FPEP (2 mM) and 3-deoxyUDP-GlcNAc (7.5 mM) in 20 mM Tris/HCl pH 7.6 containing 2 mM DTT, (b) after the addition of UDP-GlcNAc (7.5 mM). Prior to the addition of the UDP-GlcNAc to sample 2(a), the small molecular weight compounds were removed by centrifugation (Microcon-3K, Amicon), the retentate was reconstituted in buffer A and the process was repeated twice. (c) Sample 2(b) was treated with 0.2 M KOH.

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(17) The cysteine for enzyme from both sources is 115.
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(19) It is interesting to note that the chemical shift value for the EPTase-FPEP tetrahedral intermediate formed in the presence of UDP-GalNAc is slightly upfield of that formed in the presence of the normal substrate UDP-GlcNAc and that a small peak is indeed present (downfield of the major peak) in relatively the same area as seen with UDP-GlcNAc in Figure 1a. It is possible that the 3-FPEP reacts with a different nucleophile at the active site, however, upon treatment of 1c with KOH, the denatured fluoro-bound intermediate resonates at the same relative shift value as seen in 1b, and, based on the intensity of the peak, no doubt is due to C115 being labeled. In order to further explore this very interesting possibility, we plan to observe the ^{19}F-NMR of the incubation of 3-FPEP with UDP-GalNAc in the presence of our EPTase C115A mutant. If indeed, the enzyme-bound fluorinated-intermediates observed in Figures 1a and c are not present in this new experiment then they must both have been bound to C115. Alternatively, if a peak with a chemical shift identical to that observed in Figure 1c is seen, then a different nucleophile must be labeled. Should the latter be the case, proteolytic digestion coupled with HPLC-MS will be used in order to determine the labeled amino acid residue.

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