Formation of Two Enzyme-Bound Reaction Intermediate Analogs during Inactivation of UDP-GlcNAc Enolpyruvyl Transferase by (*E*)- or (*Z*)-3-Fluorophosphoenolpyruvate

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The product of the *Escherichia coli murZ* gene, UDP-GlcNAc enolpyruvyl transferase (MurZ),[1] catalyzes the first committed step in bacterial peptidoglycan synthesis and is the target of the antibiotic fosfomycin.[2-4] MurZ catalysis, which involves the transfer of an enolpyruvyl moiety from phosphoenolpyruvate (PEP) to the 3-OH group of UDP-GlcNAc with cleavage of the C–O bond of PEP, constitutes a rare biochemical transformation; the only other known enolpyruvyl transfer occurs in the shikimic acid pathway in a reaction catalyzed by 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. Although MurZ and EPSP synthase share significant sequence homology (~20% identity),[1,5] recent work suggests distinct differences in the respective mechanisms of the two enolpyruvyl transferases. In particular, while catalysis by EPSP synthase has been demonstrated to proceed through a single non-covalently-bound tetrahedral intermediate,[6] the MurZ reaction pathway (Scheme 1) has been proposed to involve a covalent enzyme-bound thiophospholactyl intermediate (**1**),[3,7,8] that precedes the formation of a tetrahedral phospholactyl UDP-GlcNAc intermediate (**2**).[9] Previously, (*E*)- and (*Z*)-isomers of 3-fluorophosphoenolpyruvate (3-F-PEP) have been synthesized and studied as potential inhibitors and mechanistic probes of PEP-utilizing enzymes.[10,11] In particular, a recent study of EPSP synthase revealed that (*Z*)-3-F-PEP, but not (*E*)-3-F-PEP, acts as a pseudosubstrate for EPSP synthase, forming a tightly-bound analog of the reaction tetrahedral intermediate that does not proceed to product.[11]

In this communication we report that both (*E*)- and (*Z*)-isomers of 3-F-PEP act as time-dependent inactivators of MurZ. In both (*E*)- and (*Z*)-3-F-PEP-inactivated preparations of MurZ, we have detected and characterized the formation of *two* stable enzyme complexes: a covalent 3-fluoro-2-phospholactyl enzyme adduct (**3**) and a non-covalently-bound 3-fluoro-2-phospholactyl UDP-GlcNAc species (**4**), both of which are fluoro analogs of the proposed intermediates in the MurZ reaction pathway.

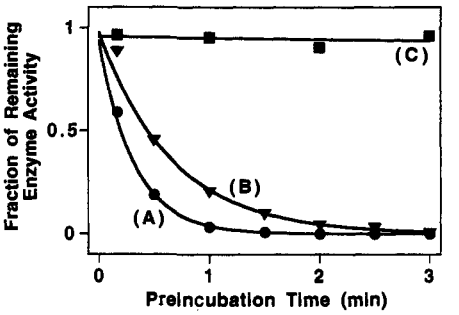

Figure 1. MurZ activity as a function of preincubation time in a reaction mix consisting of 5 μM MurZ and the following: (A) 50 μM (*E*)-3-F-PEP and 0.5 mM UDP-GlcNAc; (B) 50 μM (*Z*)-3-F-PEP and 0.5 mM UDP-GlcNAc; (C) 50 μM (*Z*)-3-F-PEP in the absence of UDP-GlcNAc.

Figure 1 shows the time-dependent inactivation of MurZ by (*E*)-3-F-PEP (curve A) and (*Z*)-3-F-PEP (curve B) in the presence of the cosubstrate, UDP-GlcNAc. On the time scale investigated neither (*E*)-3-F-PEP (data not shown) nor (*Z*)-3-F-PEP (curve C) inactivated MurZ in the absence of UDP-GlcNAc. The kinetics of inactivation were analyzed:[14] for (*E*)-3-F-PEP, $k_{inact} = 4.6 \pm 1.1$ min^{-1}, and $K_i = 38 \pm 16$ μM; for (*Z*)-3-F-PEP, $k_{inact} = 1.8 \pm 0.4$ min^{-1}, and $K_i = 36 \pm 19$ μM.

Anion-exchange HPLC[8,9] of inactivation reactions consisting of MurZ, (*E*)- or (*Z*)-3-F-PEP, and [^{14}C]UDP-GlcNAc showed no evidence of formation of an enolpyruvyl UDP-GlcNAc-like product. However, incorporation of [^{14}C]UDP-GlcNAc into a new species, **4**, with an elution volume similar to that of **2** was observed. A large-scale (~100 mg of MurZ) inactivation reaction was quenched with 0.2 N KOH after 5 min and subjected to anion-exchange HPLC for purification and subsequent NMR and mass spectrometry characterization of **4**.[15]

Figure 2A shows the ^{19}F-NMR spectrum of (*E*)-3-F-PEP-inactivated MurZ prior to quenching in order to analyze enzyme-associated species. (^{19}F-NMR spectra of (*Z*)-3-F-PEP-inactivated MurZ were equivalent; spectra not shown.) The broad resonances centered at –216 and –223 ppm represent two distinct protein-associated moieties, with chemical shifts that are characteristic of $-CH_2F$ groups. Upon quenching of the reaction with 0.2 N KOH, the spectrum changes dramatically (Figure 2B). One of the broad resonances in the spectrum in Figure 2A is replaced by a well-resolved triplet at –224.8 ppm. The chemical shift and coupling constants of this species in 0.2 N KOH correspond to those observed for purified **4**. The narrowing of the line width and resolution of F–H couplings in the base-quenched spectrum are consistent with the release from the active site of the non-covalently-bound **4**. The observation of a broad resonance centered at –217.2 ppm in the spectrum of the base-denatured enzyme (Figure 2B) is strongly suggestive of a covalent enzyme-bound adduct and is consistent with the formation of **3**.

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(14) MurZ was purifed as described in ref 8, except that a PEP "cleansing" procedure was not performed, as the excess UDP-GlcNAc present in the kinetic assays and NMR experiments resulted in the rapid conversion of bound PEP to enolpyruvyl UDP-GlcNAc product. MurZ activity was monitored using a coupled assay utilizing enolpyruvyl UDP-GlcNAc reductase, MurB (ref 12). (*E*)- and (*Z*)-3-F-PEP inactivation assays were performed at 25 °C by preincubating MurZ (5 μM), UDP-GlcNAc (0.5 mM), varying concentrations of (*E*)-3-F-PEP (18, 25, 35, 50, and 100 μM) or (*Z*)-3-F-PEP (30, 40, 60, 100, and 200 μM), and Tris buffer (50 mM, pH 8.0). At 10 s intervals, a 50 μL aliquot of the preincubation mix was diluted by addition to 950 μL of coupled assay mix (ref 12), and activity was measured within 2 min of dilution. For each concentration of (*E*)- or (*Z*)-3-F-PEP, an apparent first-order rate constant for inactivation (k_{app}) was obtained, and a plot of $1/k_{app}$ versus $1/$[(*E*)- or (*Z*)-3-F-PEP] yielded the values for k_{inact} and K_i (ref 13).

(15) ^{1}H–^{1}H DQF-COSY and ^{1}H–^{19}F HETCOR spectra were consistent with a UDP-GlcNAc-derived species with a $-CH_2F$ moiety. Negative-ion HR FAB-MS gave a peak at m/z 792.0455 consistent with **4** (calculated for $C_{20}H_{30}FN_3O_{23}P_3^-$: 792.0467).

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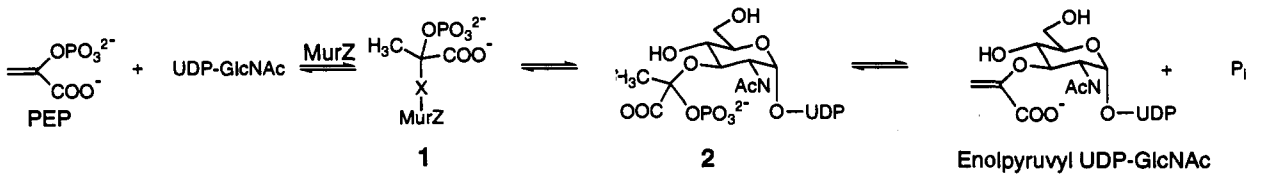
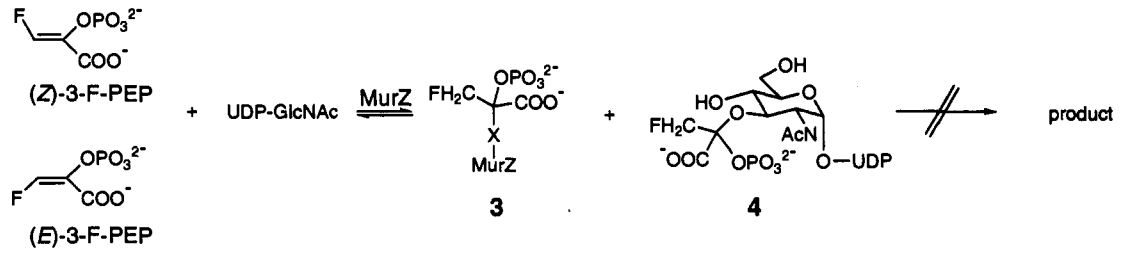
Scheme 1. Proposed Mechanism of MurZ



Scheme 2. Inactivation of MurZ by (*E*)- or (*Z*)-3-F-PEP



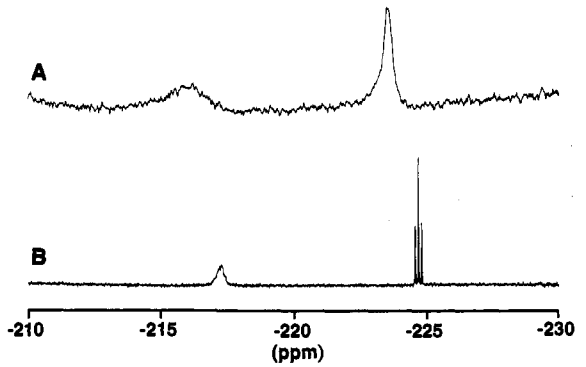


Figure 2. (A) ^{19}F-NMR spectrum (8×) of a sample containing 1.5 mM MurZ, 5 mM UDP-GlcNAc, and 2 mM (*E*)-3-F-PEP, in 50 mM Tris (pH 8.0). The sample was incubated for 40 min prior to NMR data collection. The acquisition time for spectrum A was 2 h, after which the sample was quenched by the addition of 0.2 N KOH (final concentration). (B) ^{19}F-NMR spectrum (1×) of the sample after quenching. Acquisition time for spectrum B was also 2 h. Spectra were collected on a Bruker AM400 spectrometer operating at 376 MHz and were referenced to an external standard of 1% trifluoroacetic acid (δ = −76.53 ppm) in D_2O.

Further evidence for the existence of **3** was obtained from electrospray mass spectrometry (ES-MS) of (*Z*)-3-F-PEP-inactivated MurZ samples.[16] Deconvoluted spectra showed, in addition to a peak at molecular weight 44 831 corresponding to native MurZ, a new peak corresponding to a species with molecular weight 45 015, consistent with the expected molecular weight of **3** (data not shown). Covalent linkage most likely occurs through Cys 115, which has been shown to be the site of inactivation by fosfomycin.[3,4] No protein-associated resonances were observed in the ^{19}F-NMR spectrum of a MurZ preparation that had been inactivated with fosfomycin and UDP-GlcNAc prior to the addition of (*Z*)-3-F-PEP, providing support for a covalent linkage through Cys 115 and strongly suggesting that **3** and **4** are formed at the active site of MurZ.

The observation of two forms of inactive enzyme, **3** and **4**, mimics the detection of **1** and **2** as transient intermediates in normal catalysis.[8,9] The ^{19}F-NMR spectrum of MurZ to which purified **4** has been added is similar to that observed in Figure 2A, indicating the formation of **3** from **4** within the acquisition time of the NMR experiment (12 h), and suggesting that there is an equilibrium between **3** and **4** at the active site. However, no breakdown of **4** in the forward direction is detected by ^{19}F-NMR over 12 h, reflecting a relative rate retardation of at least 10^6 compared to the breakdown of **2** in normal catalysis.[17] While these data are consistent with sequential formation of **3** followed by **4**, as has been proposed for **1** and **2** in normal catalysis, we cannot, at the present time, rule out the possibility that **3** and **4** are formed by parallel pathways in which the 3-OH of UDP-GlcNAc and the thiolate side chain of Cys 115 are in competition for direct attack on C_2 of (*E*)- or (*Z*)-3-F-PEP. Kinetic experiments to address this issue are in progress.

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(16) ES-MS was performed as described in ref 4, under enzyme-denaturing conditions (0.05% trifluoroacetic acid, water/acetonitrile).

(17) Given that the rate constant for breakdown in the forward direction of **2** is ~200 min^{-1} (ref 8), and assuming that 10% product formation could have been easily detected by ^{19}F-NMR over 12 h, the retardation of breakdown in the forward direction of **4** relative to **2** is estimated to be 10(720 min)(200 min^{-1}) > 10^6.