Stereochemical Analysis of the Tetrahedral Adduct Formed at the Active Site of UDP-GlcNAc Enolpyruvyl Transferase from the Pseudosubstrates, (*E*)- and (*Z*)-3-Fluorophosphoenolpyruvate, in D_2O

Dennis H. Kim, Watson J. Lees, and Christopher T. Walsh*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School Boston, Massachusetts 02115

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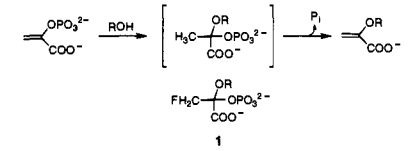
Of the remarkably diverse chemical transformations that phosphoenolpyruvate (PEP) undergoes in metabolism, the enzymic formation of an enol ether through the intact transfer of the enolpyruvyl group of PEP to a cosubstrate alcohol is notable in that there is no net electrophilic addition to C-3 of PEP. UDP-GlcNAc enolpyruvyl transferase (MurZ), which catalyzes the first committed step in bacterial peptidoglycan assembly, and 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase of the shikimic acid pathway are the two enzymes known to catalyze this unusual enopyruvyl transfer and have been the subject of considerable mechanistic investigation, in part because each enzyme constitutes the site of action of important inhibitors: the antibiotic fosfomycin inactivates MurZ,[1] and the herbicide glyphosate inhibits EPSP synthase.[2] The transient and reversible formation of a methyl group by protonation of C-3 of PEP has been demonstrated by the isolation of a tetrahedral ketal intermediate in both the EPSP synthase[3] and MurZ[4] reaction pathways by rapid quench methods, establishing an addition−elimination mechanism for enolpyruvyl transfer (Scheme 1).[5] The relative stereochemistry of the enzyme-catalyzed addition and elimination steps has been determined for both EPSP synthase and MurZ. Using stereospecifically-substituted [3-^{2}H,^{3}H]PEP as substrates for EPSP synthase and subsequently assessing the distribution of tritium into the products, the Knowles and Floss groups independently established that the coupled addition and elimination steps proceed with opposite stereochemistry; that is, if addition is *syn*, then elimination is *anti*, or the converse.[6] Recently, using (*E*)-phosphoenolbutyrate as a substrate for MurZ in D_2O, followed by analysis of the enolbutyryl kinetic product, we have determined that the pair of addition and elimination steps of the MurZ reaction also proceeds *syn/anti* or *anti/syn*.[7] With this constraint on the overall stereochemical course of the reaction in hand, the determination of the stereochemistry of addition at C-2 and C-3 of PEP would complete the stereochemical description of enzymic enolpyruvyl transfer. However, the enol ether *products* do not reveal any information regarding the absolute stereochemistry of either the addition or the elimination steps. Such information can be obtained only from analysis of the transiently-formed tetrahedral ketal reaction *intermediate*. In this Communication, we have evaluated the absolute configuration of the chiral fluoromethyl group of the tetrahedral reaction intermediate analog formed as the active site of MurZ in D_2O from the pseudosubstrates, (*E*)- and (*Z*)-3-fluorophosphoenolpyruvate (FPEP), thus revealing the face of enzyme-catalyzed addition of D^+ to FPEP and, by extension, the stereochemistry of protonation of C-3 of PEP in normal enolpyruvyl transfer catalyzed by MurZ.

We previously reported the isolation and characterization of the fluorinated tetrahedral intermediate analog **1**, in addition to a covalent enzyme−FPEP adduct, formed during UDP-GlcNAc-dependent inactivation of MurZ by (*E*)- and (*Z*)-FPEP.[8] Kinetic characterization of the processing of (*Z*)-3-fluorophosphoenolpyruvate by MurZ was strongly suggestive of the addition−elimination mechanism of Scheme 1.[9,10] Most strikingly, in contrast to the transient formation and decay of the native tetrahedral intermediate, no breakdown of **1** in the forward direction to an enolpyruvyl-like product was detected, reflecting at least a 10^6-fold electronic retardation of the elimination step.[8,9] We have taken advantage of the kinetic stability of **1** conferred by the electronegativity of the fluorine substituent, as well as the chirality of the fluoromethyl group resulting from the

Scheme 1. Addition−Elimination Mechanism of Enzymic Enolpyruvyl Transfer[a]



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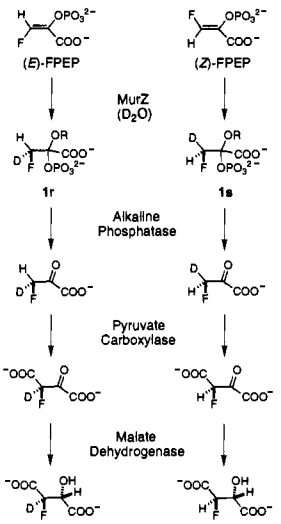
[a] MurZ: ROH = UDP-GlcNAc (3-OH). EPSP synthase: ROH = shikimate-3-phosphate (5-OH).

Scheme 2. Experimental Strategy for Stereochemical Analysis of Addition to C-3 of FPEP[a]



[a] Addition to the 2-*re* face of FPEP depicted.

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(10) Our kinetic characterization of (*Z*)-FPEP as a pseudosubstrate for MurZ (ref 9) was strongly suggestive of a branching mechanism, in which the direct pathway to product proceeds by the addition−elimination mechanism of Scheme 1. Although this has yet to be unambiguously established with the native substrate, PEP, the result in this paper addresses the stereochemistry of methyl group formation in the intermediate(s), which does not depend on branching vs sequential mechanism for MurZ (refs 4b, 9).

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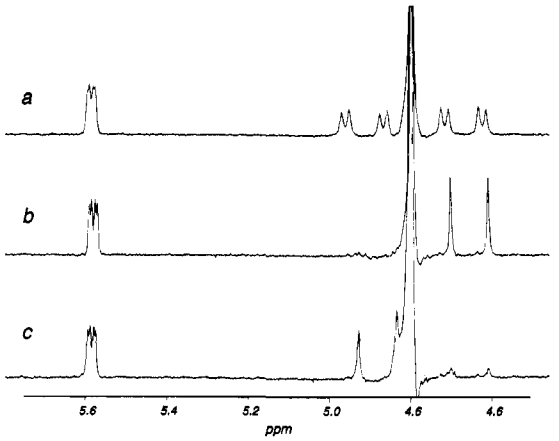

Figure 1. ^{1}H-NMR of (a) **1**, formed from (*E*)-FPEP and MurZ in H_2O; (b) **1r**, formed from (*E*)-FPEP and MurZ in D_2O; and (c) **1s**, formed from (*Z*)-FPEP and MurZ in D_2O. Each compound was formed from large-scale incubations with MurZ (0.1 mM, ~100 mg), UDP-GlcNAc (1 mM), the indicated isomer of FPEP (0.3 mM), and Tris·DCl buffer (50 mM, pD 8.0) in D_2O in a total volume of 20 mL. Isolation and purification were performed as reported previously.[9] ^{1}H-NMR spectra were collected on a Varian VXR-500 spectrometer operating at 500 MHz. The off-scale resonance at 4.8 ppm is due to HOD; the resonance at 5.6 ppm (dd, $J_{H(1)-H(2)} = 8$ Hz, $J_{H(1)-P} = 3$ Hz) is due to H-1 of the GlcNAc ring.

addition of D^+ to the FHC= terminus of FPEP, in our experimental strategy for the determination of the stereochemistry of the addition step at C-3 of FPEP (Scheme 2).

Figure 1a shows the region of the ^{1}H-NMR spectrum displaying resonances from the diastereotopic fluoromethyl hydrogens of **1**. These hydrogens give rise to the two doublet of doublets centered at 4.7 and 4.9 ppm that show characteristic J_{F-H} (46 Hz) and J_{H-H} (9 Hz) couplings. Figure 1, parts b and c, shows the same region of the ^{1}H-NMR spectrum for **1r**, isolated from the reaction of MurZ with (*E*)-FPEP in D_2O, and **1s**, isolated from the complementary reaction with (*Z*)-FPEP. Comparison of the spectrum of **1r** with that of **1** shows the disappearance of the downfield resonance arising from one of the hydrogens of the fluoromethyl group, as well as loss of the 9 Hz J_{H-H} coupling in the remaining upfield resonance, indicative of the stereospecific formation of a −CHDF group. Complementary results are obtained in the corresponding spectrum of **1s** (Figure 1c).[11]

Having established that enzyme-catalyzed D^+ additon to C-3 of FPEP is stereospecific, we proceeded to determine the chirality of the −CHDF group in **1r** and **1s** and thereby the face of addition of D^+ to FPEP (Scheme 2). Alkaline phosphatase catalyzed the breakdown of **1r** and **1s** to the corresponding enantiomers of [3-^{2}H]-3-fluoropyruvate and UDP-GlcNAc. A coupled reaction involving pyruvate carboxylase and malate dehydrogenase (MDH) was utilized for the stereospecific conversion of [3-^{2}H]-3-fluoropyruvate to (2*R*,3*R*)-3-fluoromalate that was analyzed by ^{19}F-NMR for the presence of either ^{1}H or ^{2}H attached to C-3 of (2*R*,3*R*)-3-fluoromalate.[12,17,18] The ^{19}F-NMR spectra of (2*R*,3*R*)-3-fluoromalate

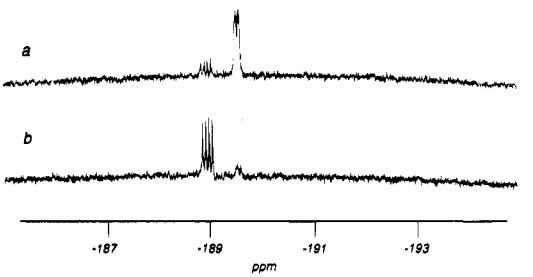

Figure 2. ^{19}F-NMR of the products of the alkaline phosphatase/pyruvate carboxylase-MDH reaction starting with (a) **1r** and (b) **1s**. Approximately 0.5 μmol (0.4 mg) of **1r** was incubated with 300 units of alkaline phosphatase (calf intestine; Boehringer Mannheim), EDTA (0.1 mM), and Tris·HCl buffer (50 mM, pH 8.0) in H_2O in a total volume of 0.5 mL at 25 °C for 1 h and filtered through a 30 kDa molecular weight cutoff filtration membrane (Amicon). The filtrate was incubated with 40 units of pyruvate carboxylase (bovine liver; Sigma), 60 units of MDH (pig heart; Boehringer Mannheim), NADH (3 mM), ATP (10 mM), acetyl-CoA (0.1 mM), $KHCO_3$ (50 mM), $MgCl_2$ (10 mM), $MnCl_2$ (2 mM), and Tris·HCl buffer (200 mM, pH 7.7) in H_2O in a total volume of 1 mL at 30 °C for 2 h. The reaction mixture was then filtered through a 30 kDa molecular weight cutoff filtration membrane, concentrated by vacuum evaporation to a volume of 0.3 mL, and then diluted with the addition of 10 mM EDTA in 0.3 mL of D_2O (added for lock). **1s** was treated identically and in parallel. ^{19}F-NMR spectra were recorded on a Bruker AM400 spectrometer operating at 376 MHz and were referenced to an external standard of 1% TFA ($\delta = -76.53$ ppm).

generated by the pyruvate carboxylase/MDH reactions are shown in Figure 2. The upfield doublet of triplets ($J_{F-H(3)} = 25$ Hz; $J_{F-D} = 7$ Hz, $I = 1$ for deuterium) at −189.5 ppm, predominant in the spectrum of Figure 2a, arises from the deuterated species, [3-^{2}H]-3-fluoromalate, while the downfield doublet of doublets ($J_{F-H(2)} = 50$ Hz; $J_{F-H(3)} = 25$ Hz) at −189 ppm, predominant in the spectrum of Figure 2b, is due to the [3-^{1}H]-3-fluoromalate species.[12]

The results from the application of the strategy outlined in Scheme 2 dictate the assignment of the absolute configuration of **1r** at the fluoromethyl group[18] as *R* and the configuration of **1s** at the fluoromethyl group as *S* (as shown in Scheme 2), from which it follows that addition of D^+ was to the 2-*re* face of FPEP. Extending these findings to the normal physiological MurZ reaction, we conclude that the proton addition step at C-3 of PEP proceeds at the same face of the double bond, the 2-*si* face.[19]

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(11) The low-intensity downfield doublet in Figure 1b results from a minor impurity of (*Z*)-FPEP (12%) in the (*E*)-FPEP material, attenuated 2.5-fold (refs 8, 9) by the kinetic preference of MurZ for the (*E*) isomer. Similarly, the low-intensity upfield doublet in Figure 1b is due to the presence of 5% (*E*)-FPEP in the (*Z*)-FPEP preparation, in this case amplified 2.5-fold by the kinetic preference of MurZ for the (*E*) isomer.

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(18) 3-Fluoropyruvate was previously shown to be a substrate for pyruvate carboxylase (ref 13), but the stereospecificity of the reaction with 3-fluoropyruvate had not been established. ^{19}F-NMR analysis of the pyruvate carboxylase/MDH reaction with 3-fluoropyruvate showed the formation of only the 2*R*,3*R* diastereomer of fluoromalate (data not shown), which was distinguished from the 2*R*,3*S* diastereomer by the distinctive $J_{F-H(2)}$ couplings of the 2*R*,3*R* species (refs 12, 14). Because pyruvate carboxylase is known to proceed with retention of stereochemistry at C-3 (ref 15), we concluded that pyruvate carboxylase stereospecifically abstracts the *pro-S* hydrogen of 3-fluoropyruvate (i.e., chiral recognition of the fluoromethyl group (ref 16)), as has been found to be the case for a related biotin-dependent carboxylase, transcarboxylase (refs 12, 17).

(19) Note that the fluorine substituent results in an inversion of the *re/si* designation.