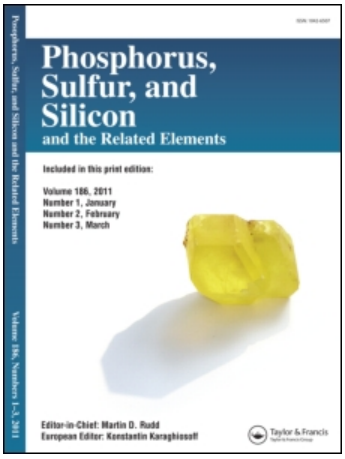

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Insights from ^{31}P NMR Studies of Substrate and Inhibitor Complexes with EPSP Synthase

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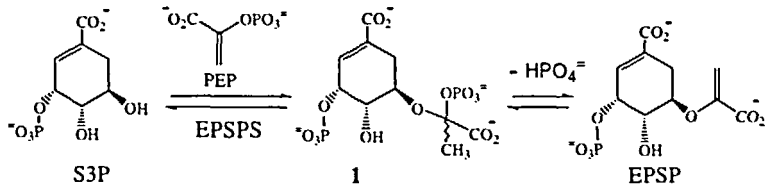
Attempts to observe the formation of binary and ternary enzyme complexes by ^{31}P NMR have uncovered several unusual side reactions both on and off the catalytic pathway of this system.

Keywords: ^{31}P NMR; EPSP synthase; S3P; PEP; 3-Fluoro-PEP

INTRODUCTION

Spectroscopic studies of substrate and inhibitor complexes with EPSP (5-enolpyruvoylshikimate 3-phosphate) synthase (EPSPS, E.C. 2.5.1.19) have provided important corroborative evidence for our current view of this enzyme's chemical and kinetic mechanism. As the biological target for the commercially successful herbicide, glyphosate,[1] EPSPS catalyzes an unusual transfer reaction of the



carboxyvinyl portion of phosphoenolpyruvate (PEP) regiospecifically to the 5-OH of shikimate 3-phosphate (S3P) forming EPSP and inorganic phosphate (P_i).[1,2] This enzyme proceeds through a single, kinetically competent,[3] tightly bound[4] tetrahedral intermediate **1**. Since each of these substrates and **1** contain phosphate functionality, ^{31}P NMR has been particularly useful to probe binding interactions at the individual subsites and to understand the reaction dynamics of this important enzyme.

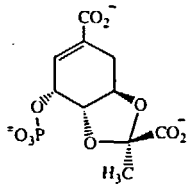
^{31}P NMR STUDIES OF BINARY SUBSTRATE COMPLEXES

S3P forms a reasonably tight (K_d = 7 ± 1.2 μM)[5] binary complex with EPSPS. The formation of an S3P binary complex (3.9 ppm) is readily apparent from the downfield chemical shift of the ^{31}P NMR signal for S3P's 3-phosphate group which occurs under conditions where >95% of S3P is enzyme-bound, relative to S3P in the absence of enzyme (3.54 ppm).[6] In contrast, while EPSP forms a tighter binary complex (K_d = 1 ± 0.01 μM)[5] than S3P, no significant chemical shift is observed for the EPSP 3-phosphate signal at 3.22 ppm even when enzyme is present in large excess. Spectroscopic studies of bound EPSP are complicated by a known side reaction (hydrolysis of EPSP to S3P and pyruvate) that occurs quite readily at the enzyme concentrations needed for NMR studies,[7] and the difficulty in distinguishing EPSP*EPSPS from its ternary complex with P_i. PEP forms a relatively weak binary complex with enzyme (K_d = 390 ± 15 μM),[8] and no significant change in the ^{31}P NMR signal (-0.88 ppm) for PEP occurs in the presence of excess enzyme.

^{31}P NMR STUDIES WITH EPSPS AT EQUILIBRIUM

The detection, isolation and structural elucidation of **1** was accomplished under internal equilibrium conditions from a detailed understanding of the pre-steady state kinetics of this system and access to radiolabeled substrates. Efforts to observe the dynamic formation of **1** spectroscopically using ^{13}C enriched PEP and EPSP were complicated by two side reactions that occurred off the catalytic pathway which were facilitated by the larger enzyme concentrations employed for NMR studies. The hydrolysis of EPSP to S3P and pyruvate has already been described. The EPSP cyclic ketal **2** was a

more interesting side reaction product which was first detected using ^{13}C and ^{31}P NMR studies of EPSPS at equilibrium.[9] Prolonged incubation of EPSPS with excess substrates at equilibrium produces **2** as the only detectable new shikimate species present. Interestingly, although **2** forms off the normal catalytic pathway, the optimum production of **2** occurs when **1** is maximized with enzyme. Ketal **2** forms a weaker binary complex with EPSPS than EPSP (K_d = 24 ± 8 μM),[8] and no significant change in the ^{31}P NMR signal (2.18 ppm) for **2** occurs in the presence of excess enzyme.



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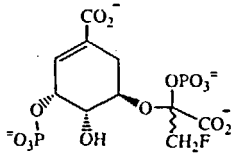

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^{31}P NMR STUDIES WITH EPSPS and 3-FLUORO-PEP

As a competitive inhibitor for PEP,[10] 3-F-PEP unexpectedly forms a detectable binary complex with EPSPS by ^{31}P NMR (0.38 ppm).[11] Upon the addition of S3P, this complex produces the fluoro tetrahedral intermediate analog **3**, as first observed by ^{31}P NMR, and later corroborated[12] by ^{13}C and ^{19}F NMR. Two new significantly broadened F-PEP phosphate signals (-0.01, 0.50 ppm) were observed by ^{31}P NMR, indicating a tight interaction with enzyme and a change in local environment relative to the F-PEP binary complex. Analog **3** can be isolated intact from enzyme and has been fully characterized by NMR and mass spectrometry. The ^{31}P NMR spectrum of isolated **3** (δ = 0.38 and -5.54 ppm) is essentially identical to that of isolated **1**. Kinetic studies confirmed that **3** forms a very tight complex with EPSPS ($K_{d(est)}$ = 600 pM).[12] As such, **3** is the most potent inhibitor of EPSPS identified to date. Interestingly, isolated **3** is much more stable to acid than **1**. Enzyme-bound **3** is also very stable, and EPSPS is unable to convert **3** to a fluoro-EPSP product. Thus, 3-F-PEP functions as an unusual pseudo-substrate for EPSPS. Similar results have recently been reported in studies of F-PEP turnover with UDP-GlcNAc enolpyruvyl transferase, the only other enzyme known to

catalyze carboxyvinyl transfer.[13] These studies with F-PEP have thus helped identify a unified chemical mechanism for the unusual chemistry catalyzed by both enzymes.[13]

CONCLUSIONS

NMR investigations of EPSPS have uncovered multiple new reaction products and greatly expanded our understanding of the basic chemistry of this important enzyme system.

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