perature (307 K) for the amide NH signals.

In summary, the large cleft-like shapes and variety of chemical linings presented by the new structures permit their use for the selective chelation of sizable, functionally complex molecules. Their facile assembly augurs well for the development of a new generation of medicinal agents wherein *concave synthetic surfaces are tailored to enfold smaller, convex targets.*[12] This would represent a reversal of roles for current strategies in which the smaller synthetic agent is directed at the folds of a biological macromolecule.

Acknowledgment. We thank the National Institutes of Health for financial support and Dr. J. Huff for advice. The fission fragment mass spectrometric analyses were performed by Brian Chait of The Rockefeller University Mass Spectrometric Research Resource supported by the Division of Research Resources, NIH.

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Isolation and Structural Elucidation of the Tetrahedral Intermediate in the EPSP Synthase Enzymatic Pathway

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Received June 22, 1988

EPSP (5-enolpyruvoylshikimate-3-phosphate) synthase is an enzyme in the shikimic acid pathway that catalyzes the unusual transfer of an enolpyruvoyl moiety from PEP to S3P with the elimination of inorganic phosphate. The enzyme is the target of the commercially important herbicide glyphosate, *N*-(phosphonomethyl)glycine.[1,2] In this report, we describe the isolation and structure determination of the tetrahedral intermediate formed at the active site of the enzyme from the nucleophilic attack of the 5-OH of the S3P on the C-2 position of PEP as shown in Scheme I.

Our previous work has demonstrated the presence of an acid-labile intermediate in the reaction pathway.[3,4] Rapid quench kinetics afforded the complete kinetic description of the reaction showing that a single intermediate was formed within 5 ms on the enzyme and decayed to form products (EPSP and phosphate) over the next 50 ms. Although the data were strongly suggestive of a tetrahedral intermediate, the structure was not definitively established because it decomposed under acidic quench conditions to form pyruvate and S3P.

Scheme I

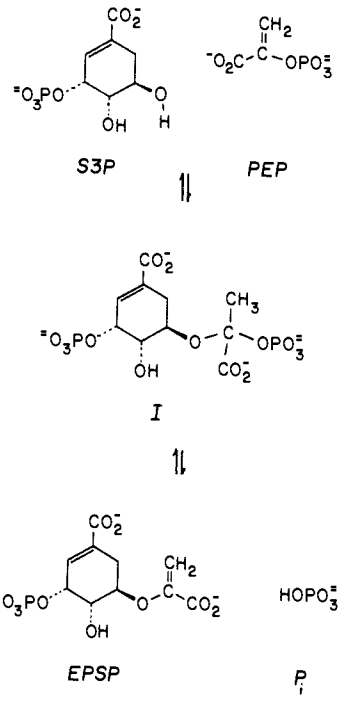

Table I. Synthesis of the Intermediate[a]

radiolabel	[S3P]	[PEP]	[P_i]	rx time	% intermediate	% yield
[^{14}C]S3P	4 μM	2 mM	10 mM	5 s	33	90
[^{14}C]PEP	100 μM	3.5 μM		10 ms	15	60
[^{32}P]PEP	100 μM	3.5 μM		10 ms	12	50

[a] The indicated concentrations of substrates were incubated with 10 μM enzyme for the specified time,[5] and then the reaction was stopped by mixing with neat triethylamine. The percentage conversion of radiolabel into intermediate was quantitated by HPLC using a Synchropak AX-100 column with a continuous flow radioactivity detector. Details of the methods for rapid mixing were described previously.[4] Rationale for design of the experiments to form the intermediate under single turnover (10 ms) or equilibrium (5 s) conditions and calculations of the expected yields were based upon previous rapid quench kinetic and equilibrium measurements.[4]

We have now discovered that if the enzyme is denatured rapidly by quenching under mildly basic conditions with neat triethylamine, the intermediate is stable and can be isolated by ion exchange HPLC.

The first test of the identity of the intermediate was conducted with [^{14}C]S3P, [^{14}C]PEP, or [^{32}P]PEP to synthesize the intermediate on the enzyme under conditions described previously.[4,5] In each case, the efficiency of the incorporation of radiolabel into the intermediate was 50–90% of the theoretical yield (Table I). These results demonstrated that the intermediate contained the shikimate ring donated by S3P and both the enolpyruvoyl and the phosphate moieties donated by PEP.

The enzyme catalyzed the decomposition of the isolated intermediate, thus reinforcing our identification of the adduct as a reaction intermediate.

The structure of the intermediate was confirmed by ^{1}H NMR, ^{31}P NMR, and ^{13}C NMR. The required quantity (300 μg) of the intermediate was synthesized enzymatically by mixing enzyme with equimolar [^{14}C]S3P and high concentrations of [^{13}C]-2-PEP

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(5) All enzyme reactions were performed at 20 °C in a buffer containing 50 mM HEPES and 50 mM KCl at pH 7.0.

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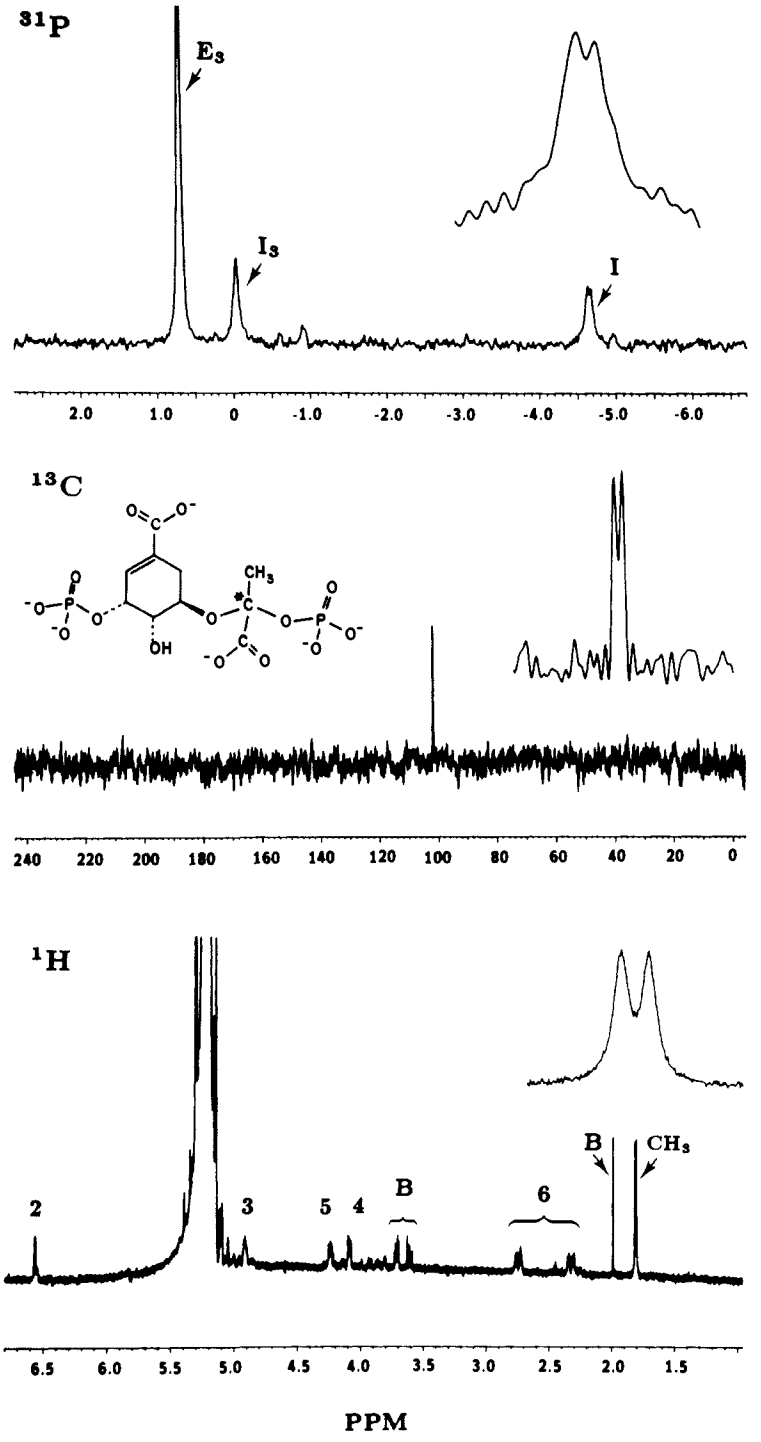

Figure 1. NMR Spectra of the Tetrahedral Intermediate. Middle inset: structure of the intermediate; *C denotes the position of the ^{13}C label. **^{31}P NMR Spectrum.** The phosphorus-31 spectrum of the mixture of EPSP (75%) and intermediate (25%) was obtained on a Bruker WM-360 at 145.81 MHz in D_2O. Chemical shifts reported in ppm relative to trimethyl phosphate. The spectrum was obtained with WALTZ ^1H decoupling and a 3-s delay between pulses. The phosphates attached to the 3 position on the ring of EPSP and the intermediate are designated by (E_3) and (I_3), respectively. The phosphate attached to the tetrahedral carbon is designated by I. Inset: expansion showing splitting of the phosphate (at −4.6 ppm) attached to tetrahedral carbon. **^{13}C NMR Spectrum.** The carbon-13 spectrum of the purified intermediate was obtained on a Bruker AM-500 at 125.78 MHz with ~3 M K_2HPO_4 in D_2O. The spectrum was obtained with WALTZ ^1H decoupling and a 3-s delay between pulses. Chemical shifts are reported in ppm relative to tetramethylsilane. Dioxane was used as a reference (δ = 67.6 ppm). Inset: expansion showing splitting of the tetrahedral carbon (at 101.7 ppm) by phosphate. **^1H NMR Spectrum.** The proton spectra were obtained on a Bruker AM-500 at 500.13 MHz in ~3 M phosphate buffer in D_2O. Chemical shifts are in ppm relative to acetone (δ = 2.17 ppm). The resonances for the ring protons are designated by the conventional numbering of the ring (2-6). The resonances denoted by B were contaminants that were present in the ~3 M phosphate. The methyl resonance is denoted by CH_3. Inset: expansion showing splitting of the methyl proton resonances (at 1.8 ppm) by the tetrahedral carbon-13. **Methods.** To synthesize the intermediate, enzyme (10 mL at 800 μM) was mixed with an equal volume of substrates (800 μM [^{14}C]S3P, 1 mM [^{13}C]-2-PEP, and 5 mM phosphate) and allowed to react for 5 s, and then the reaction was stopped by mixing with neat triethylamine in a rapid quench apparatus.[4,6] Denatured protein was removed from the quenched reaction mixture by centrifugation, and the intermediate was isolated from the aqueous layer. Chromatography on a Mono-Q (LKB/Pharmacia) anion-exchange column (0.15–0.35 M ammonium bicarbonate gradient) produced a mixture of EPSP (75%), and the intermediate (25%) resolved from S3P, PEP, and phosphate. Separation of the EPSP from the intermediate was achieved with a Synchropak AX-100 column (isocratic 300 mM potassium phosphate, pH 8.0). Samples were lyophilized and then resuspended in D_2O. Because it was not possible to remove the phosphate after this column, ^{13}C and ^1H spectra were obtained in the presence of ~3 M phosphate, and the ^{31}P spectrum was obtained on the mixture containing EPSP (75%) and intermediate (25%).

(2 mM) and phosphate (5 mM). Under these conditions the following internal equilibrium is established at the active site of the enzyme, with 30% of the enzyme-bound material in the form of the intermediate.[4] The intermediate was isolated from the

$$E\cdot S3P\cdot PEP \rightleftarrows E\cdot I \rightleftarrows E\cdot EPSP\cdot P_i$$

enzyme reaction mixture as described in the legend to Figure 1 and analyzed by NMR.

A proton decoupled ^{31}P NMR spectrum was obtained on the mixture of EPSP (75%) and intermediate (25%). Three major phosphate signals were observed (Figure 1). The two downfield signals correspond to the phosphates in the 3 position of the shikimate ring for both EPSP and the intermediate. A third signal at −4.6 ppm corresponds to the phosphate attached to the tetrahedral carbon of the intermediate. This phosphorus resonance is split into a doublet by coupling to the ^{13}C at the tetrahedral center of the intermediate (see inset), providing definitive assignment.

The proton decoupled ^{13}C NMR spectrum of the purified intermediate (Figure 1) revealed the presence of a single peak having a chemical shift of 101.7 ppm, suggestive of a tetrahedral carbon bearing two oxygens. This resonance was split into a doublet with a coupling constant, $^2J_{CP} = -7$ Hz. This coupling constant was equal, within experimental error (±0.4 Hz), to that observed in the phosphorus spectrum. This carbon–phosphorus coupling demonstrates that the phosphate is attached to the tetrahedral carbon in the intermediate.

A final confirmation of the structure of the intermediate was provided by obtaining a ^1H NMR spectrum (Figure 1). The spectrum was very similar to that previously reported for EPSP,[7] with the addition of a distinctive methyl group of the intermediate at 1.8 ppm that was split into a doublet by coupling to the ^{13}C at the tetrahedral center (see inset).

These observations, coupled to our previous rapid quench kinetics,[4] provide definitive identification of the tetrahedral adduct as a true intermediate in the EPSP synthase reaction pathway. We can now conclude with confidence that the reaction proceeds by an addition–elimination mechanism involving the nucleophilic attack of the 5-OH of S3P on the C-2 of PEP (Scheme I). Although this pathway has been suggested previously,[4,7,8] the current work provides the first unequivocal evidence for an addition–elimination mechanism and lays to rest the controversy surrounding the suggestion of a covalent enolpyruvoyl–enzyme intermediate.[9-11]

Although there are numerous examples of covalent intermediates formed during nucleophilic catalysis, this study provides a unique example of the isolation of a stable, noncovalent enzyme intermediate that is formed and broken down catalytically by the enzyme. This is unusual in that enzymes are most often described by their ability to stabilize an unstable intermediate. Further work is in progress to determine the pH dependence of breakdown of the intermediate in solution and to quantitate fully the mechanistic implications of such an intermediate. Out data suggest that analogues of the tetrahedral intermediate will bind tightly to the enzyme.

Acknowledgment. We thank Professor William P. Jencks for the suggestion that the tetrahedral intermediate should be stable at high pH.

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Observation of Reactive *o*-Quinodimethanes by Flow NMR

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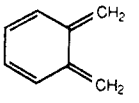
Received October 26, 1987

The reactive molecules, *o*-xylylene (**1**),[1] the parent benzenoid *o*-quinodimethane (*o*-QDM), and its derivatives, have been molecules of considerable theoretical[2,3] and experimental[3-7] interest since **1** was first postulated in 1957 by Cava.[8] We have recently



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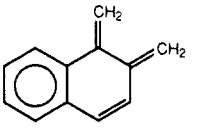
observed several *o*-QDM's by UV–vis spectroscopy and have used the stopped-flow technique to study their rates of dimerization.[9] The first reported ^1H NMR of an *o*-xylylene derivative is that of 2,2-dimethyl-2*H*-indene (2,2-dimethylisoindene) by Dolbier and Michl.[10] In this communication we report the detection of 1,2-dimethylene-1,2-dihydronaphthalene (**2**) and **1** in the presence



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of its stable dimers by the technique of flow ^1H NMR.[11-18] The flow NMR technique allows detection of short-lived species and usefully complements UV–vis techniques because of its diagnostic nature.

Recently, it was reported that *o*-QDM's can be generated by fluoride ion induced 1,4-elimination from [*o*-((trimethylsilyl)-methyl)benzyl]trimethylammonium halides.[7,19] We have found this reaction to be very fast,[9] and thus this method provides an excellent means for the generation of reactive *o*-quinodimethanes under flow NMR conditions because fast, quantitative formation of the transient species is desired.

Our flow NMR apparatus was patterned after that of Fyfe[11,13-18,20] who has pioneered the use of flow NMR in the detection and characterization of reactive intermediates. Because successful detection of a reactive intermediate requires the proper

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