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Observation by ^{13}C NMR of the EPSP Synthase Tetrahedral Intermediate Bound to the Enzyme Active Site

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ABSTRACT: Direct observation of the tetrahedral intermediate in the EPSP synthase reaction pathway was provided by ^{13}C NMR by examining the species bound to the enzyme active site under internal equilibrium conditions and using [2-^{13}C]PEP as a spectroscopic probe. The tetrahedral center of the intermediate bound to the enzyme gave a unique signal appearing at 104 ppm. Separate signals were observed for free EPSP (152 ppm) and EPSP bound to the enzyme in a ternary complex with phosphate (161 ppm). These peak assignments account for our quantitation of the species bound to the enzyme and liberated upon quenching with either triethylamine or base. A comparison of quenching with acid, base, or triethylamine was conducted; the intermediate could be isolated by quenching with either triethylamine or 0.2 N KOH, allowing direct quantitation of the species bound to the enzyme. After long times of incubation during the NMR measurement, a signal at 107 ppm appeared. The compound giving rise to this resonance was isolated and identified as an EPSP ketal [Leo et al. (1990) *J. Am. Chem. Soc.* (in press)]. The rate of formation of the EPSP ketal was very slow, 3.3×10^{-5} s^{-1}, establishing that it is a side product of the normal enzymatic reaction, probably arising as a breakdown product of the tetrahedral intermediate. A slow formation of pyruvate was also observed and is attributable to the enzymatic hydrolysis of EPSP, with 5% of the enzyme sites occupied by EPSP and hydrolyzing EPSP at a rate of 4.7×10^{-4} s^{-1}. To look for additional signals that might arise from a covalent adduct which has been postulated to arise from reaction of enzyme with PEP, an NMR experiment was performed with an analogue of S3P lacking the 4- and 5-hydroxyl groups. Enzyme was incubated with 4,5-dideoxy-S3P and [2-^{13}C]PEP and examined by ^{13}C NMR. Only the signal for PEP was observed. All of these results reaffirm our identification of the tetrahedral species as the only observable intermediate in the EPSP synthase reaction.

EPSP synthase is an enzyme in the shikimic acid pathway which catalyzes the unusual transfer of an enolpyruvoyl moiety from PEP to S3P with the elimination of inorganic phosphate. It is the target enzyme of the commercially important herbicide glyphosate [*N*-(phosphonomethyl)glycine] (Franz, 1985; Steinrucken & Amrhein, 1980). In previous papers, we have described the isolation and structure determination of a 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 and have provided a complete kinetic description of the EPSP synthase reaction pathway (Anderson et al., 1988b,c). Recently, we have established the kinetic properties of this intermediate in solution and when added back to enzyme (Anderson & Johnson, 1990).

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Although the previous work led to definitive identification of the tetrahedral intermediate and kinetic analysis argued for a single intermediate, it was important to determine whether we could obtain direct evidence by ^{13}C NMR for the intermediate bound to the active site of the enzyme and to look for additional intermediates. In the present study we have provided a better quantitation of the species bound to the enzyme under conditions of internal equilibrium, which provides a firm basis for interpretation of the ^{13}C NMR spectra. In the course of this work, we have established that an additional species, EPSP ketal I (Leo et al., 1990), is formed too slowly to participate as an intermediate during catalysis and is therefore a side product.

While the present paper was under review, another paper describing the analysis of species bound to the enzyme by ^{13}C NMR methods appeared (Barlow et al., 1989). The current paper resolves the controversy suggested by Barlow et al. involving their misassignment of the EPSP ketal and free PEP as a "enzyme-free intermediates" and their misinterpretation of previously published data.

Materials and Methods

Enzyme Purification. EPSP synthase was isolated from a cloned *Escherichia coli* strain that overproduces the enzyme (Rogers et al., 1983). The enzyme was purified as described previously.

Chemicals. Shikimate 3-phosphate (S3P) was synthesized enzymatically by treatment of shikimic acid (Sigma Chemical Co., St. Louis, MO) with shikimate kinase (Millar et al., 1986) and purified as described previously. The [^{14}C]S3P was synthesized and purified in a similar manner starting with uniformly labeled shikimic acid (New England Nuclear, Boston, MA). The specific activity of the [^{14}C]shikimic acid was 19 mCi/mmol. Unlabeled PEP was purchased from Sigma. Radiolabeled [^{14}C]PEP was obtained from Amersham (Arlington Heights, IL; specific activity 32 mCi/mm). The [2-^{13}C]PEP (monopotassium salt) was purchased from MSD Isotopes (Montreal, Canada). The 4,5-dideoxy-S3P used in this study was synthesized by Dr. Michael J. Miller (Monsanto). All buffers and other reagents employed were of the highest commercial purity. Millipore ultrapure water was used for all solutions. All experiments were conducted at 20 °C in HEPES buffer (50 mM) containing potassium chloride (50 mM) at pH 7.0.

Rapid Quench Experiments. The rapid quench experiments were performed as described previously. Samples were quenched with either 0.2 N KOH and 0.2 N HCl, or 33% triethylamine (final concentrations) to obtain the results described in Table I.

HPLC Analysis. The substrates and products were quantitated by HPLC using a continuous flow radioactivity detector. The HPLC separation was performed by using a Mono Q anion-exchange column (HR 5/5, Pharmacia, Piscataway, NJ) with a flow rate of 2 mL/min. Gradient separation was employed where solvent A is ultrapure water and solvent B is 0.5 M triethylammonium bicarbonate. The linear gradient program was as follows: 35–100% solvent B in 6 min, hold at 100% solvent B for 5 min, recycle to 35% solvent B in 5 min, and reequilibrate at 35% solvent B for 6 min. The HPLC effluent from the column was then mixed with liquid scintillation cocktail (Atomlight, New England Nuclear) with a flow rate of 6 mL/min. Radioactivity was monitored continuously by using a Flo-One radioactivity detector (Radiomatic Instruments, Tampa, FL). The analysis system was automated by the use of a Waters WISP (Milford, MA) autosampler.

Fluorescence Titration Measurements. Equilibrium fluorescence measurements made on the EPSP ketal were made in a manner as described previously (Anderson et al., 1988a).

NMR Spectroscopy. The buffer employed for all NMR analyses was 50 mM HEPES and 50 mM KCl in D_2O at pD 7.0 with 10% glycerol. All data were collected on a Bruker AM-500 spectrometer resonating at 125.78 MHz with WALTZ ^1H decoupling. Chemical shifts are reported in parts per million relative to tetramethylsilane. Dioxane in the above buffer was used as a reference ($\delta = 67.6$). Our chemical shifts differ by ~3 ppm from those reported by Barlow et al. (1989); this difference could be due to the 0.1 M phosphate present in their samples. The number of scans ranged from 4000 to 35000, and 64K data points were collected. The pulse width was 10 μs. A 10-mm Bruker VSP broadband probe was employed for all ^{13}C NMR experiments, and the temperature was maintained at 6 °C.

Results and Discussion

Quantitation of Internal Equilibrium. We previously reported the isolation of the tetrahedral intermediate after quenching the enzymatic reaction with triethylamine. The intermediate was isolated by quenching the enzyme within 15 ms after E·S3P was mixed with PEP under single-turnover conditions or after 5 s of reaction when an internal equilibrium at the active site of the enzyme was established between S3P, intermediate, and EPSP by including high concentrations of PEP and P_i (Anderson et al., 1988b,c) according to the scheme

$$\text{E·S3P} + \text{PEP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E·S3P·PEP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{E·I} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{E·EPSP·P}_i \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{E·EPSP} + \text{P}_i$$

Our previous analysis of the equilibrium distribution of species bound to the active site was limited by our inability to resolve chromatographically the tetrahedral intermediate from EPSP and the decomposition of the intermediate under acidic quench conditions. We therefore repeated our analysis of the internal equilibrium distribution by rapidly denaturing the enzyme with triethylamine and analyzing the radiolabeled products by HPLC. An improved HPLC separation was developed using a Mono Q 5/5 anion-exchange column with triethylammonium bicarbonate buffer. In our earlier experiments, two chromatographic steps were required: the first was a Mono Q 5/5 anion-exchange column using ammonium bicarbonate, and the second was an AX-100 Synchropak column and phosphate buffer (Anderson et al., 1988c). The second step was necessary to separate EPSP from intermediate since they co-eluted in the first step. Surprisingly, when we substituted triethylammonium bicarbonate for ammonium bicarbonate, base-line separation of all reaction products was obtained with the Mono Q column.

The distribution of species present under internal equilibrium conditions is illustrated in Figure 1, which shows the HPLC profile of radioactivity with the radiolabel contained in the shikimate ring, after 10 μM enzyme, 4 μM S3P, 2 mM PEP, and 15 mM phosphate were mixed. The relative percentages of S3P, intermediate, and EPSP observed experimentally are in close agreement with the proportions predicted by the previously determined rate constants. Thus, at equilibrium, 4% of the sites are occupied by S3P and PEP, 39% by intermediate, 52% by EPSP and P_i, and 5% by EPSP alone.

The enzymatic reaction could be quenched with either triethylamine or 0.2 N KOH to liberate the intermediate. A comparison of the results of quenching the enzyme with either

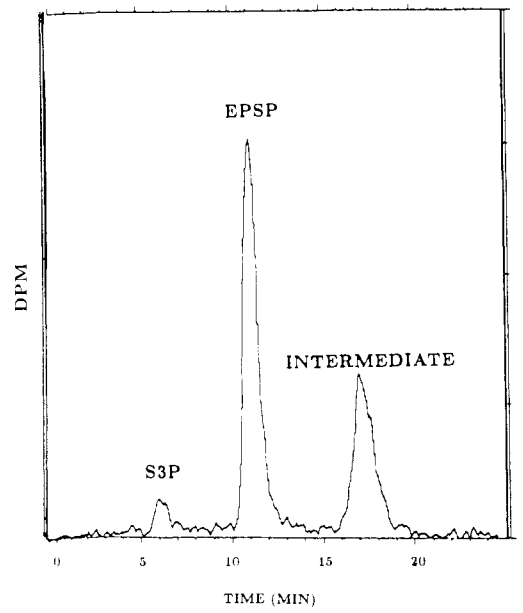


FIGURE 1: HPLC quantitation of internal equilibrium at the enzyme active site between S3P, intermediate, and EPSP. Separation of [^{14}C]S3P, intermediate, and EPSP was performed on a Mono Q 5/5 anion-exchange column using triethylammonium bicarbonate gradient (0.175–0.50 M) at pH 7.8 with radioactivity monitoring (1000 dpm full scale). The flow rate was 2 mL/min. The calculated and observed product percentages are as follows: S3P (calculated, 4%; observed, 6%); intermediate (calculated, 40%; observed, 33%); and EPSP (calculated, 56%; observed, 61%). The calculated percentages are predicted by previously determined rate constants.

Table I: Distribution of Products Observed[a]

product	predicted[b]	acid	base	triethylamine
PEP	2.20	2.20	2.20	2.32
EPSP	0.80	0.81	0.85	0.79
intermediate	0.50		0.45	0.39
pyruvate		0.49		

[a] A solution containing EPSP synthase (4 μM) and S3P (100 μM) was mixed with [^{14}C]PEP (3.5 μM), and the reaction was then quenched after 15 ms by mixing with either 0.2 N HCl and 0.2 N KOH, or 33% triethylamine (all concentrations are those after mixing). The distribution of label appearing in pyruvate, PEP, or EPSP is given in micromolar units of concentration during the enzymatic reaction. Conditions: 50 mM HEPES, 50 mM KCl, pH 7.0, 20 °C. [b] Calculated by numerical integration of the reaction time course according to rate constants previously published (Anderson et al., 1988b).

acid, base, or triethylamine is shown in Table I. In these experiments, a preformed enzyme–S3P complex was mixed with radiolabeled PEP for 15 ms and then mixed with the quenching agent. As shown in the table, the intermediate (radiolabeled in the phospholactyl moiety) breaks down exclusively to pyruvate in acid. The intermediate is stable under basic conditions. Similar results were obtained under internal equilibrium conditions by utilizing radiolabel in the shikimate ring of S3P and allowing equilibrium to be established in 5 s in the presence of high concentrations of unlabeled PEP and P_i.

Barlow et al. (1989) have suggested that the observed tetrahedral intermediate could have been formed by the attack of phosphate on a covalently bound enzyme intermediate during the "nonphysiological" quench conditions. However, our quantitative data demonstrating a high, constant yield of the tetrahedral intermediate in the presence or absence of phosphate argue against such superficial dismissal of the rapid quench results.

In a recently completed study, we have addressed the stability of the intermediate in solution and in the presence of enzyme (Anderson & Johnson, 1990). The intermediate has a half-life in solution of 45 min at pH 7.0, but binds to the enzyme with a second-order rate constant of 5×10^7 M^{-1} s^{-1}, reacting to form the normal enzymatic products of the forward and reverse reactions. At pH $\geq$12 the half-life of the intermediate in solution is >48 h.

Table II: Assignment of Chemical Species and Rates of Formation[a]

species	chemical shift (ppm)	rate of formation (s^{-1})
$intermediate_{bound}$	104	1200
EPSP ketal	107	0.000 033
PEP_{free}	148	10
$EPSP_{free}$	152	60
$EPSP_{bound}$	161	320
pyruvate	204	0.000 024

[a] The assignments are given for each species observed by ^{13}C NMR analysis of the internal equilibrium mixture containing S3P, [2-^{13}C]-PEP, and phosphate as described in Figure 2. In addition, the rates of formation of each species were measured at 20 °C in 50 mM HEPES, pH 7.0, and 50 mM KCl; kinetic constants for the intermediate, PEP, and EPSP are from Anderson et al. (1988b). The rate of EPSP ketal formation could be attributed to reaction of the enzyme bound intermediate to form the ketal with a rate constant of 1×10^{-4} s^{-1} and 40% of the enzyme sites occupied by intermediate. The rate of pyruvate formation can be accounted for by the measured rate of EPSP hydrolysis (4.7×10^{-4} s^{-1}, Anderson et al., 1988b) with 5% of the enzyme sites in E·EPSP state in the internal equilibrium.

Observation of the Intermediate Bound to the Enzyme. By establishing that an internal equilibrium at the enzyme active site could be maintained and quantitated, these studies provided the conditions necessary to examine the species bound to the enzyme directly by NMR methods. In our previous work, synthesis of the intermediate from [2-^{13}C]PEP provided a unique carbon-13 resonance due to the tetrahedral center in the intermediate. According to our quantitation of the internal equilibrium at 20 °C, we expect approximately 39% of the sites to be occupied by the intermediate and 57% by EPSP at equilibrium. Accordingly, use of the [2-^{13}C]PEP in a reaction with S3P under equilibrium conditions at a high enzyme concentration should allow direct observation of the intermediate, EPSP, and possibly other species bound to the enzyme.

This experiment required that millimolar concentration of enzyme be used to provide the sensitivity necessary to detect the intermediate by ^{13}C NMR. The experiment was conducted in three stages as shown in Figure 2. Spectrum a shows the downfield region of a proton-decoupled natural abundance ^{13}C NMR of the enzyme alone (1.3 mM). This shows the standard carbonyl and aromatic resonances for the protein backbone. Spectrum b shows the results of the addition of [2-^{13}C]PEP (6 mM) and phosphate (15 mM) to the enzyme. Two additional resonances appear: a large peak corresponding to C-2 of PEP at 148 ppm and a small peak at 144 ppm that was due to a minor unidentified contaminant present in the commercial preparation of [2-^{13}C]PEP (data not shown). Spectra c and (d) show the results obtained after the addition of S3P (3 mM) to the solution containing enzyme, [2-^{13}C]PEP, and phosphate; spectrum c was obtained within 1.0 h after the addition of S3P to the enzyme–PEP mixture, while spectrum d was collected over 4.5 h.

The following expected resonances were observed: 104 (tetrahedral intermediate), 148 (PEP), and 152 ppm (EPSP). Three additional resonances were observed at 107, 161, and 204 ppm. Comparison of spectra c and d demonstrates that the peaks at 107 and 204 ppm arise late in the reaction time and therefore are unimportant for catalytic turnover. The resonance giving rise to the 107 ppm peak was shown to be

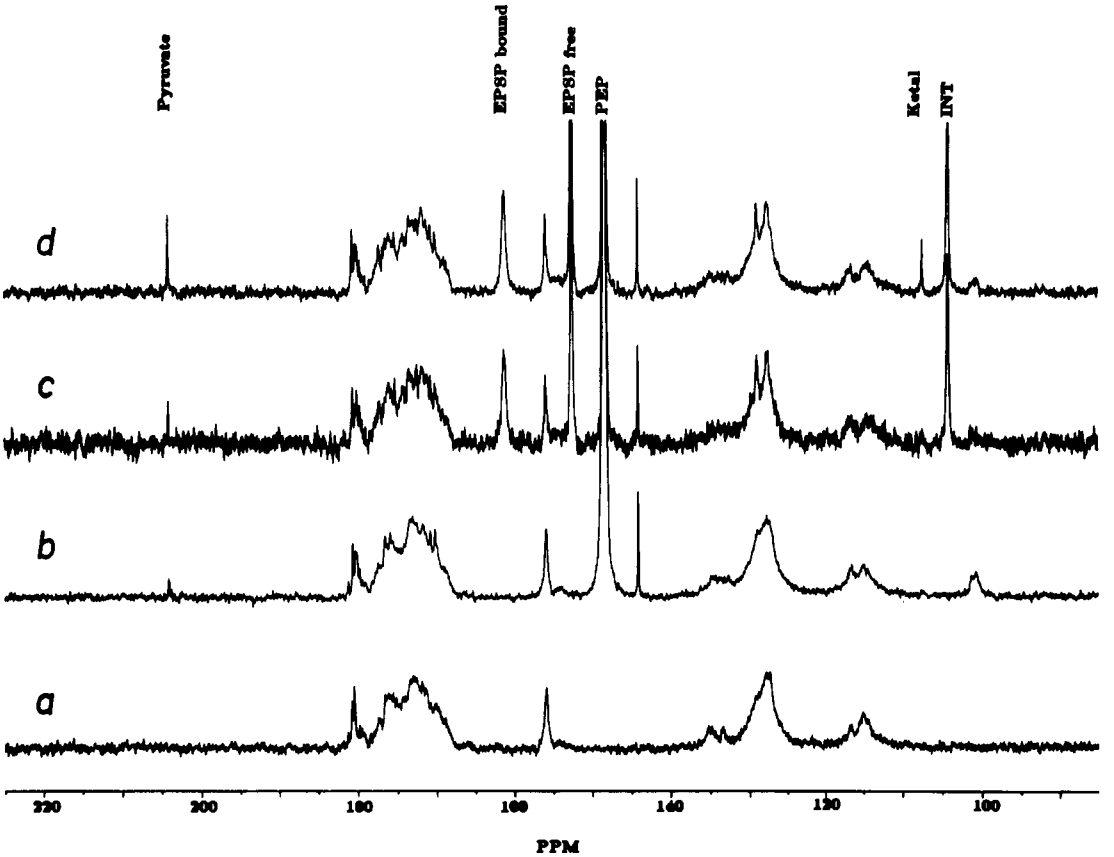


FIGURE 2: ^{13}C NMR spectrum of the tetrahedral intermediate bound to the enzyme active site. (a) Downfield region of proton-decoupled natural abundance ^{13}C NMR spectrum of EPSP synthase (1.3 mM) in 50 mM HEPES and 50 mM KCl in D_2O containing 10% glycerol at pH 7.0. (b) Downfield region of the proton-decoupled ^{13}C NMR spectrum of EPSP synthase (1.3 mM) plus [2-^{13}C]PEP (6 mM) and potassium phosphate (15 mM). Two new resonances were observed upon the addition of PEP. A large peak appearing at 148 ppm was assigned to the carbon 2-position of PEP and a small resonance at 144 ppm which was shown to arise from a minor contaminant present in the commercial preparation of [2-^{13}C]PEP. In addition, a broad resonance of low intensity was observed at 101 ppm, which could be due to natural abundance at C-3 of PEP. (c, d) Downfield region of the proton-decoupled ^{13}C NMR spectrum of EPSP synthase (1.3 mM) with [2-^{13}C]PEP (6 mM) and S3P (3 mM). Spectrum c was obtained within 1 h after the addition of S3P (4000 scans), while spectrum d was obtained after 4.5 h (17 538 scans). The following new resonances were observed: 104 ppm (intermediate); 107 ppm (a slowly formed side product which is an EPSP ketal (Leo et al., 1989); 152 ppm (EPSP free in solution); 161 ppm (EPSP bound in a ternary complex with enzyme and phosphate); and 204 ppm (pyruvate formed from very slow enzymatic hydrolysis of EPSP). All spectra were obtained on a Bruker AM-500 resonating at 125.78 MHz for ^{13}C. The spectrum was obtained with WALTZ ^1H decoupling. Chemical shifts are reported in parts per million relative to tetramethylsilane. Dioxane was used as a reference (δ = 67.6). 64K data points were collected. The pulse width was 10 μs. All data were obtained with a 10-mm Bruker VSP broadband probe and Fourier transformed with a line broadening of 7 Hz.

the EPSP ketal, I (Leo et al., 1990). The peak at 204 ppm corresponded to pyruvate which formed over long periods of time at a rate of 2.4×10^{-5} s^{-1} due to very slow enzymatic hydrolysis of EPSP. Table II summarizes our peak assignments and the rates of formation of each species identified.

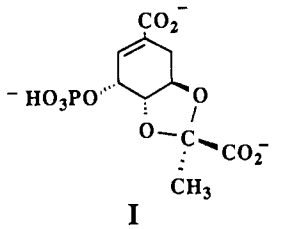
The resonance at 161 ppm could be explained if there are separate resonances for EPSP *free* in solution and for EPSP *bound* to enzyme in both binary (E·EPSP) and ternary (E·EPSP·P_i) complexes. The chemical shift for EPSP bound to enzyme in the binary complex in the absence of phosphate is 156 ppm (data not shown). Under the conditions for the experiment described in Figure 2, the excess S3P would result in a concentration of 1.7 mM EPSP free in solution. According to our kinetic and equilibrium measurements, greater than 90% of the EPSP bound to enzyme would be in the form of a ternary complex with phosphate at the active site. To test further the suggestion of separate resonances for free EPSP and EPSP bound in a ternary complex, we designed an NMR experiment with PEP equimolar to enzyme (1.6 mM) and in slight excess over S3P (1.5 mM). Under these conditions, all of the EPSP (K_d of 1 μM) formed would be bound to the enzyme in the ternary complex, due to the tight binding for EPSP and the high concentration of phosphate (15 mM). When the experiment was performed, the resonance at 152 ppm, corresponding to EPSP free in solution, was no longer observed (data not shown), while the resonance at 161 ppm remained. Since we must quantitatively account for all species known to be bound to the enzyme, this experiment establishes that the resonance at 161 ppm is due to the EPSP bound to the enzyme in a ternary complex with phosphate.

It is interesting to note that the resonance for the bound EPSP in the ternary complex was shifted ~9 ppm downfield relative to free EPSP, while the binary E·EPSP complex was shifted by only 4 ppm. One possible explanation of the larger downfield shift could be the environment at the active site required to react EPSP with phosphate to form the intermediate. Alternatively, EPSP may be in rapid equilibrium with a carbonium ion species as a very reactive intermediate on the pathway toward formation of the tetrahedral intermediate. In either case, one cannot argue that the peak at 161 ppm is due to an entirely new intermediate because we must account for the liberation of EPSP upon quenching the enzyme with either acid or base.

The line widths at half-height for free EPSP (10 Hz), bound EPSP in a ternary complex (65 Hz), and bound intermediate (27 Hz) are in qualitative agreement with the chemical exchange lifetimes as calculated from the kinetic constants measured at 20 °C. These resonances are all in the slow

chemical exchange limit. Although the line widths are somewhat narrower than calculated from the kinetic constants, the NMR was performed at 6 °C in the presence of 10% glycerol, while the kinetic constants were determined at 20 °C in the absence of glycerol. The lower temperature and the glycerol should lead to slower rates of chemical exchange and therefore narrower line widths. Moreover, the narrower line width observed for free versus bound EPSP supports our identification of species bound to the enzyme.

EPSP Ketal: "Enzyme-Free Intermediate" or Side Product? The compound giving rise to the resonance at 107 ppm has been isolated and shown to be the EPSP ketal, I (Leo et al., 1990). The isolated EPSP ketal bound to the enzyme with



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a K_d of 3 μM as determined by an equilibrium fluorescence titration experiment (data not shown). Because formation of the EPSP ketal was not observed in single-turnover experiments (Anderson et al., 1988b), it is not an intermediate in the reaction.

To further explore whether the EPSP ketal is likely to be a side product rather than a reaction intermediate, we measured its rate of formation. The EPSP ketal is readily separated from S3P, intermediate, and EPSP via anion-exchange chromatography on a Mono Q 5/5 column using a triethylammonium bicarbonate gradient. The rate of formation of the EPSP ketal was monitored by using radiolabeled S3P and excess PEP under conditions that maintain the internal equilibrium at the active enzyme site. The observed rate, 3.3×10^{-5} s^{-1}, is more than a millionfold slower than the rate of formation of EPSP from the intermediate (320 s^{-1}) (Table II). This clearly demonstrates that the EPSP ketal is a side product of the normal reaction. Moreover, this experiment emphasizes the importance of quantitative rate measurements since a qualitative interpretation of the NMR data could lead one to postulate a more complicated mechanism with the ketal as an enzyme intermediate. In particular, Barlow et al. (1989) misassigned the EPSP ketal as an "enzyme-free" form of the intermediate and were unable to distinguish among several rather complex mechanisms of reaction. They also misassigned what we have identified as free PEP as C-2 of the "enzyme-free intermediate".

We can explain how the ketal might form at the active site by an S_N2 displacement of the phosphate at the tetrahedral center by the 4-hydroxyl on the shikimate ring. This is a slow process because of the required alignment of the groups at the active site in a favorable orientation for the displacement to occur, although other modes of ketal formation are possible. The EPSP ketal also forms as one of the breakdown products of the tetrahedral intermediate in the absence of enzyme, but its rate of formation in solution is too slow ($\sim 4 \times 10^{-5}$ s^{-1}) to account for the observed formation of the EPSP ketal in the presence of the enzyme. That is, because the binding of the intermediate to the enzyme is tight and rapid (Anderson & Johnson, 1990), virtually no intermediate exists free in solution in the presence of enzyme, and because the rates of formation of the EPSP ketal in the presence and absence of enzyme are comparable, the ketal must be formed at the enzyme site.

Reaction with Dideoxy-S3P. To look for additional signals that might arise from other potential intermediates in the reaction, an NMR experiment was performed by using 4,5-dideoxy-S3P (dideoxy-S3P), an analogue of S3P lacking the 4- and 5-hydroxyl groups. Previous work by Anton et al. (1983) led to the suggestion that a carbonium ion or a covalent enzyme species was formed by reaction of PEP at the active site in the presence of dideoxy-S3P. An experiment similar to that described in Figure 2 was performed but with dideoxy-S3P and ^{13}C-labeled PEP (data not shown). The design of this experiment was firmly based upon previously measured dissociation constants for dideoxy-S3P and PEP to ensure that the enzyme sites were fully occupied (Anderson et al., 1988b). However, the only resonances observed were C-2 of PEP at 148 ppm and the minor contaminant at 144 ppm, indicating that no products of reaction of PEP at the enzyme site could be detected under these conditions. This is in accord with our previous acid quench results where no pyruvate was seen after short times of incubation of enzyme, ddS3P, and PEP (Anderson et al., 1988b). After extended incubation times, a small amount of pyruvate was observed. These data further substantiate our identification of the tetrahedral intermediate in the EPSP synthase reaction pathway.

Concluding Remarks

This study illustrates the power of a combination of kinetic and spectroscopic methods but also emphasizes the importance of direct, quantitative measurement of the events occurring at the enzyme active site. In particular, the time dependence of the reactions and the quantitation of species bound to the active site by rapid quench methods has allowed us to design and interpret NMR experiments and to establish the reaction pathway to the exclusion of alternate models. If we had relied solely on NMR techniques, we could not have assigned the resonance observed at 104 ppm definitively as the tetrahedral adduct of S3P and PEP and we would have been misled by the observation of the EPSP ketal at 107 ppm. In fact, our current NMR data (Figure 2) were obtained before our isolation and identification of the tetrahedral intermediate (Anderson et al., 1988c), and at that time the NMR data were uninterpretable. Although resonances in this region of the spectrum (100–110 ppm) are suggestive of a tetrahedral carbon bearing two oxygens, none of the possible reaction pathways can be distinguished solely by observation of these resonances.

The recent publication by Barlow et al. (1989) demonstrates the limitations of attempting to identify enzyme intermediates on the basis of ^{13}C NMR data in the absence of auxiliary information. These authors dismiss previously published rapid quench data as "nonphysiological"; however, without this information they are unable to assign resonances to either of the two species that they observed. Nonetheless, the need to account for the species observed by rapid quench methods was implicit in their tentative assignment of bound EPSP and the tetrahedral intermediate.

Saturation transfer experiments performed by Barlow et al. were interpreted to support the authors' assignment of the EPSP ketal as an "enzyme-free intermediate". Moreover, analysis of the line widths led Barlow et al. to suggest that "the enzyme-free intermediate dissociates slowly from the enzyme and does not reassociate". The tetrahedral intermediate isolated in solution rebinds to the enzyme quite rapidly, with a second-order rate constant of 5×10^7 M^{-1} s^{-1} and an estimated K_d of 0.04 nM (Anderson & Johnson, 1990). On the basis of this knowledge, the observation of an "enzyme-free

intermediate" by NMR measurements in the presence of enzyme is highly implausible.

Barlow et al. (1989) also report a nonspecific phosphatase, which must be a contaminant in their preparations because 800 μM S3P and 1 mM glyphosate only inhibit the rate by 80%; these concentrations are 40 times higher than should be required to see 98% inhibition of EPSP synthase (Anderson et al., 1988a).

Other side reactions were also shown to occur, involving the hydrolysis of EPSP at the active site to produce pyruvate and breakdown of the intermediate to form the ketal. These reactions are also slow; the rate of EPSP hydrolysis in the absence of phosphate is 4.7×10^{-4} s^{-1} (Anderson et al., 1988b); with 5% of the sites occupied by EPSP under internal equilibrium conditions, we predict a rate of pyruvate formation of 2.4×10^{-5} s^{-1}, which is consistent with the observed rate. These slow side reactions add little new mechanistic information to illuminate the events at the active site. For example, the hydrolysis of EPSP is probably a function of the protonation of the substrate at the active site followed by attack by water rather than phosphate. The formation of the EPSP ketal might have implications for the geometry of the intermediate bound to the active site, but the slowness of the reaction and uncertainty of the pathway of formation of the ketal could lead one to question the validity of such conclusions. Rate measurements are necessary to extract mechanistic conclusions from structural data, and, certainly, the converse is equally true.

Current data provide overwhelming support to conclude that the EPSP synthase reaction proceeds by a simple addition–elimination mechanism via the formation of a tetrahedral intermediate, as originally proposed in the pioneering work from Sprinson's laboratory (Bondinell et al., 1971).

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Sequence-Specific ^{1}H NMR Assignments and Secondary Structure of Eglin c†

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ABSTRACT: Sequence-specific nuclear magnetic resonance assignments were obtained for eglin c, a polypeptide inhibitor of the granulocytic proteinases elastase and cathepsin G and some other proteinases. The protein consists of a single polypeptide chain of 70 residues. All proton resonances were assigned except for some labile protons of arginine side chains. The patterns of nuclear Overhauser enhancements and coupling constants and the observation of slow hydrogen exchange were used to characterize the secondary structure of the protein. The results indicate that the solution structure of the free inhibitor is very similar to the crystal structure reported for the same protein in the complex with subtilisin Carlsberg. However, a part of the binding loop seems to have a significantly different conformation in the free protein.

Eglin c is a proteinase inhibitor from the leech *Hirudo medicinalis* (Seemüller et al., 1977). It inhibits the granulocytic proteinases elastase and cathepsin G and some other proteinases such as chymotrypsin and subtilisin. Its inhibitory properties make the protein an interesting molecule for pharmaceutical application as antiinflammatory agent. Eglin c is a member of the potato inhibitor 1 family. It consists of a single polypeptide chain with 70 residues. When eglin c was first detected, it was isolated together with hirudin and eglin b, which were identified as protein fractions a and b, respectively, while eglin c was fraction c. Eglins b and c differ by a single amino acid exchange at position 35 (histidine in eglin b, tyrosine in eglin c). Although eglin is lacking disulfide bonds, it is very stable against denaturation by heat (Seemüller et al., 1977, 1980). The crystal structure of the protein has been determined in the complex with subtilisin Carlsberg (McPhalen et al., 1985, 1987; Bode et al., 1986, 1987). The structure of the homologous serine proteinase inhibitor CI-2 from barley seeds has been determined in the complex with proteinases by X-ray crystallography (McPhalen & James,

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