The Catalytic Mechanism of EPSP Synthase Revisited[†]

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ABSTRACT: Recent analysis of EPSP synthase by solid-state NMR has led to the postulation of a new enzyme reaction pathway and raised once again the question of an intermediate species covalently bound to the enzyme [Studelska, D., McDowell, L., Espe, M., Klug, C., and Schaefer, J. (1997) *Biochemistry 36*, 15555−15560]. Therefore, we have reexamined the mechanism of the reaction catalyzed by EPSP synthase and analyzed the reaction products formed under the conditions used in preparing samples for solid-state NMR. Single-turnover experiments were carried out using both [1-^{14}C]- and [^{32}P]PEP showing the formation and decay of the previously proposed tetrahedral intermediate species on a time scale comparable with the disappearance of substrate and formation of product, thus unequivocally establishing the kinetic competence. The possible presence of a covalently bound enzyme intermediate species was also investigated, using SDS−PAGE and Centricon concentration analysis of the quenched reaction samples. No covalently bound enzyme intermediates were observed during the reaction. An enzyme assay was also performed repeating the conditions used in sample preparation for the solid-state NMR studies. We show that under these conditions, total turnover of substrates to products was observed within 45 s at −30 °C prior to freezing and lyophilization. Following lyophilization, the samples were stored at −20 °C and analyzed over a period of 21 days. We observed the conversion of the product EPSP into the side product, a cyclic EPSP ketal, and the breakdown product, pyruvate. Thus, the new species reported by solid-state NMR can be accounted for by previously characterized reaction products and side products formed during sample preparation and upon incubation in the solid-state. Our conclusions are also supported by the solution and solid-state NMR studies recently reported [Jakeman et al. (1998) *Biochemistry 37*, 12012−12019]. These results once again highlight the importance of kinetic competence as a criterion to be used in defining enzyme intermediates and point to the errors in interpretation of results when the time dependence of formation of the proposed intermediates is not considered.

EPSP[1] synthase (5-enolpyruvoylshikimate 3-phosphate) synthase catalyzes the transfer of the enolpyruvoyl moiety from phosphoenolpyruvate (PEP) to shikimate 3-phosphate (S3P) to form the products EPSP and inorganic phosphate (*1*). The enzyme is inhibited by the commercial herbicide Glyphosate, which competes with PEP in the presence of enzyme-bound S3P (*2*). Although the order of substrate binding has been suggested to be random (*3*), the *kinetically preferred* reaction pathway, as shown by substrate trapping experiments, was demonstrated to be ordered, with S3P binding first and PEP second while product release showed the release of phosphate first and then EPSP (*4*). Dead-end complexes formed from E*S3P*P_i were also described and characterized by this model. The reaction proceeds with C−O bond cleavage of PEP, as well as exchange of the vinylic protons of PEP (*5*−*7*). While most enzymatic reactions utilizing PEP as a substrate involve cleavage of the high-energy P−O bond ($\Delta G^{o'} = -14.8$ kcal/mol), there are only three other known enzymatic reactions which proceed with C−O bond cleavage of PEP: UDP−GlcNAc enolpyruvoyl transferase (*Mur Z*; also called *Mur A*); 3-deoxy-D-*manno*-2-octulosonate 8-phosphate (Kdo8P) synthase; and 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase (*8*, *9*). The *MurZ* reaction was observed to proceed through two kinetically competent intermediates, one enzyme-bound phospholactoyl and one tetrahedral intermediate (*10*, *11*), although subsequent analysis has indicated that the enzyme-bound phospholactoyl species may involve a branched pathway (*12*).

Previous studies in this laboratory have established the catalytic mechanism for EPSP synthase as shown in Figure 1. The reaction proceeds by an addition−elimination mechanism through the tetrahedral intermediate **I**. In a series of papers, the intermediate was first observed as a transient

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[1] Abbreviations: Å, angstrom; DAHP, 3-deoxy-D-*arabino*-heptulonsonate 7-phosphate; DTT, dithiothreitol; EPSP, 5-enolpyruvoylshikimate 3-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; Int., intermediate; KCl, potassium chloride; Kdo8P, 3-deoxy-D-*manno*-2-octulosonic acid 8-phosphate; MOPS, 3-*N*-morpholinopropanesulfonic acid; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol); PEP, phosphoenolpyruvate; P_i, inorganic phosphate; ppm, parts per million; RC mutant, EPSP synthase mutant (containing N94S, I113M, F172W, and W289Q); REDOR, rotational echo double resonance; S3P, shikimate 3-phosphate.

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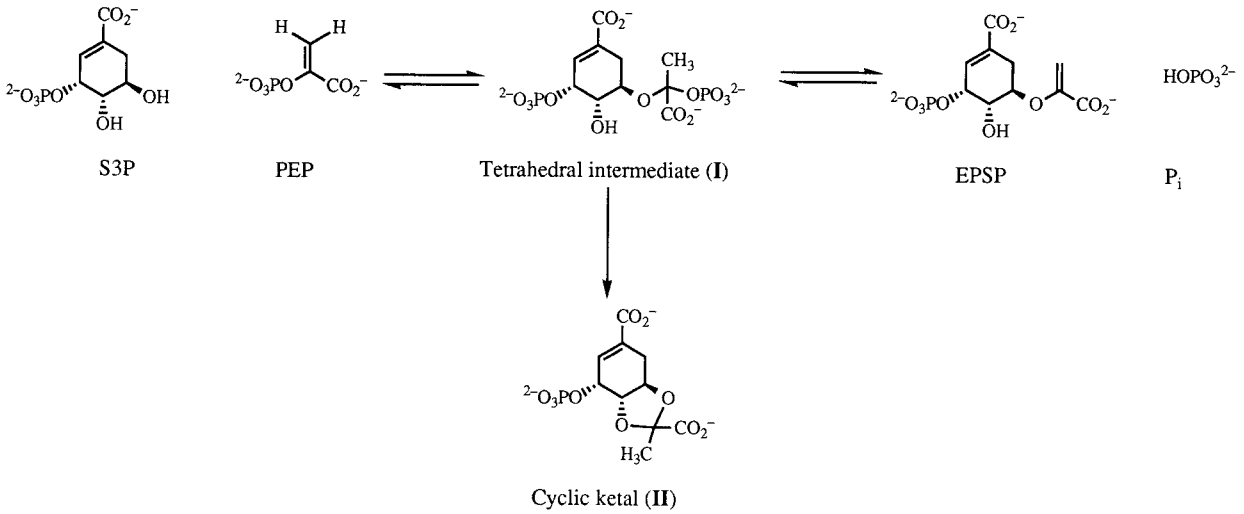

FIGURE 1: Currently accepted proposal for the mechanism of the enzyme reaction catalyzed by EPSP synthase.

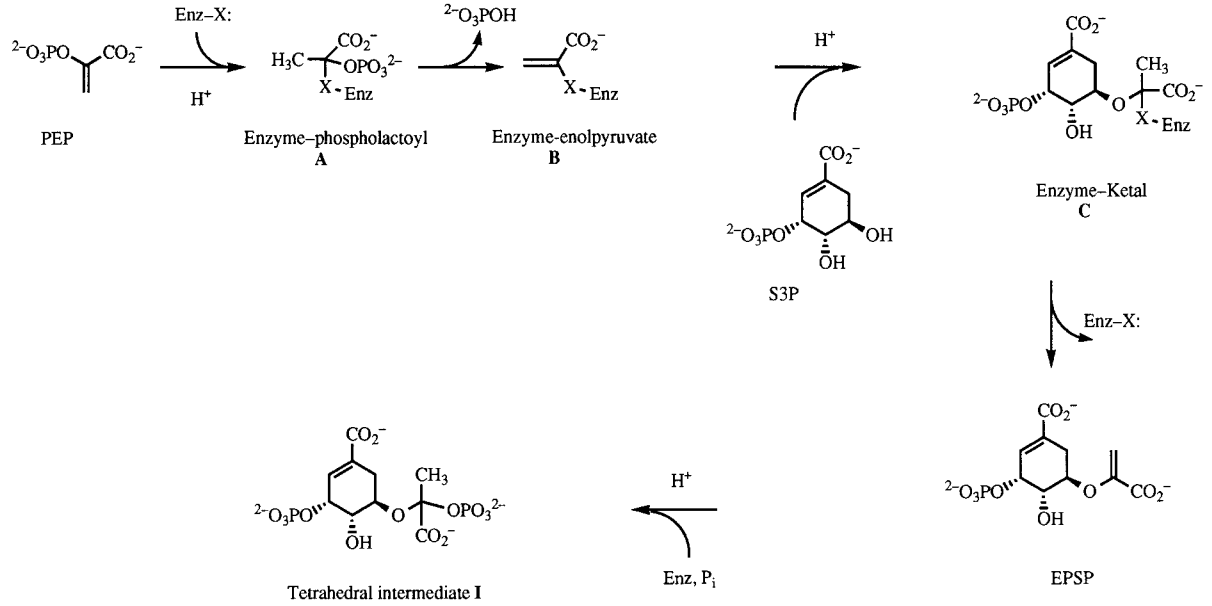

FIGURE 2: Proposal of mechanism based on solid-state NMR studies conducted by Schaefer.

species in single-turnover experiments, proving its kinetic competence as an intermediate (*4*). The intermediate was then isolated, its structure was established (*13*), and its reaction when added back to the enzyme was examined (*14*). We then showed the intermediate bound to the enzyme under internal equilibrium conditions using ^{13}C NMR methods (*15*) as have others (*16*). After longer times at internal equilibrium, it was also shown that a side product, the EPSP ketal, forms after long times of incubation on the time scale required to collect data for NMR (*14*, *15*, *24*). The EPSP ketal was once mistaken for a new enzyme intermediate in solution NMR studies (*16*), but it is now accepted as a side product formed at a rate a millionfold slower than catalysis (*15*). Thus, there is a wealth of evidence to support the identification of the intermediate **I** formed during the reaction catalyzed by EPSP synthase and to define side products.

Recent analysis by solid-state NMR has called this mechanism into question (*17*). By using EPSP synthase mutant enzymes, lyophilized enzymes, and low temperature, to slow enzyme turnover, Schaefer and co-workers have proposed two new intermediate species, covalently bound to the enzyme as illustrated in Figure 2. These conclusions and the formulation of an alternative mechanism are based upon analysis of lyophilized samples of enzyme mixed with substrates using solid-state NMR. In preparation for the solid-state NMR studies, the enzyme was first cooled to −30 °C in methanolic solutions containing PEG 8000 and trehalose. These reagents serve as protectants against the lyophilization and cryogenic conditions. The solution is then mixed with an equimolar concentration of substrates. Samples were incubated for several minutes, then frozen, lyophilized, and then analyzed by solid-state NMR, requiring several days of data collection. The results from studying a mutant form of EPSP synthase termed an RC mutant (the RC or "recombinant circle" mutant contains four mutations: N94S, I113M, F172W, and W289Q) led to the identification of resonances corresponding to the intermediate **I** which had been previously characterized. However, the authors concluded that species **I** was formed *not* as an intermediate in the reaction but rather as a side product appearing only after the reaction products EPSP and phosphate have formed. Furthermore, samples obtained using a double mutant form

of EPSP synthase (F172W, W289Q) or wild-type enzyme showed the formation of a "new" ^{13}C resonance at 155 ppm, followed by its conversion to another new resonance at 108 ppm. These resonances at 155 and 108 ppm were assigned to novel covalent enzyme intermediates: enzyme-bound enolpyruvoyl intermediate **B** and enzyme-bound ketal **C** (Figure 2), respectively. It was speculated that these were previously unseen due to conditions used by rapid chemical quench techniques.

Although the alternate mechanistic pathway outlined in Figure 2 is inconsistent with the large body of evidence already defining the reaction pathway (*24*), we reinvestigated the reaction mechanism. In this report, we describe the *direct* observation of the transient formation and decay of the intermediate **I** in a single-turnover reaction. Furthermore, to test the assumptions inherent in the solid-state NMR analysis, we characterize the time dependence of product formation under the conditions used to prepare the samples at −30 °C and after freezing and lyophilization. We show that the enzymatic reaction was completed prior to the beginning of the NMR analysis and that the "new" species identified by solid-state NMR actually correspond to the product, EPSP, and the EPSP ketal side product. Our conclusions are also supported by a recent analysis of the EPSP synthase reaction by solution-phase and solid-state NMR studies (*18*).

EXPERIMENTAL PROCEDURES

General Methods. EPSP synthase was isolated from a cloned *Escherichia coli* strain as previously described (*4*). Shikimate 3-phosphate (S3P) was synthesized enzymatically by treatment of shikimic acid (Sigma Chemical Co.) with shikimate kinase (*19*). S3P was purified and standardized as previously described (*4*). [1-^{14}C]PEP was synthesized enzymatically by treatment of [1-^{14}C]pyruvate (Amersham International) with pyruvate phosphate dikinase (PPDK) (a generous gift from Dr. Debra Dunaway-Mariano). [1-^{14}C]-PEP was purified by chromatography on Q-Sepharose eluted with a gradient of triethylammonium bicarbonate. The solution of PEP was lyophilized to dryness and used as the triethylammonium salt. The specific activity of the [1-^{14}C]-pyruvate was 28 mCi/ mmol. [^{32}P]PEP was synthesized enzymatically by treatment of EPSP with $H_3{}^{32}PO_4$ (Amersham International) with EPSP synthase. [^{32}P]PEP was purified by chromatography on Q-Sepharose eluted with a gradient of triethylammonium bicarbonate. The solution of PEP was lyophilized to dryness and used as the triethylammonium salt. The specific activity of the $H_3{}^{32}PO_4$ was 10 mCi/mL. EPSP was synthesized enzymatically from S3P and PEP (Sigma Chemical Co.) with EPSP synthase. Purification and standardization were accomplished in a manner similar to that described for S3P.

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 and 5 mM β-mercaptoethanol except where stated.

Determination of Active Site Concentration. The concentration of active sites was determined as previously described (*20*).

Rapid Quench Experiments. The rapid quench experiments were performed using an apparatus designed and built by Johnson (*21*) and manufactured by KinTek Corp. (Austin, TX; www.kintek-corp.com). The reaction was initiated by mixing the enzyme solution (15 μL) with the radiolabeled substrates (15 μL). The reaction mixture was then quenched by mixing with 67 μL of 0.29 M KOH to give a final concentration of 0.2 M KOH. In all cases, the concentrations of enzyme and substrates cited in the text are those after mixing and during the enzymatic reaction.

Manual quench samples were prepared (*17*) by dissolving EPSP synthase (150 μM) in 2 mM MOPS, pH 7.2, 1% (w/v) PEG 8000, 1 mM DTT, and 20 mM trehalose, to a total volume of 15 μL. This solution was stored on ice. Ice-cold methanol (5 μL) was added to the solution and then cooled to −30 °C. The substrates, S3P and [1-^{14}C]PEP, were dissolved in 2 mM MOPS, pH 7.2, 1% (w/v) PEG 8000, 1 mM DTT, and 20 mM trehalose each to a final concentration of 75 μM (15 μL), and stored on ice. Fifteen microliters of ice-cold methanol was added to the substrates and then cooled to −30 °C. The substrates were mixed with the enzyme solutions and quenched with KOH (67 μL) at 45 s. The samples were mixed thoroughly and maintained at −30 °C, prior to analysis. A control experiment was performed in which the enzyme was mixed with the quenching agent (KOH) prior to addition of substrate at −30 °C. Aliquots of each sample were then analyzed by HPLC as described below.

Further samples were prepared and frozen. The frozen samples were lyophilized and stored at −20 °C. The aliquots of lyophilized reaction material were resuspended in 0.2 N KOH, prior to analysis by HPLC, to prevent the enzymatic reaction proceeding upon warming up of the reaction material to room temperature.

HPLC Analysis. The substrates and products were quantified by HPLC with on-line radioactivity detection. The HPLC separation was performed on a Mono-Q anion exchange column (HR 5/5, Pharmacia, Piscataway, NJ) with a flow rate of 1 mL/min. The following gradient separation was employed where solvent A is ultrapure water and solvent B is 1.0 M triethylammonium bicarbonate. The linear gradient program was as follows: 25−35% B (0−5 min), hold at 35% (5−15 min), 100% B (15−20 min), recycle to 25% B (20−30 min). The HPLC eluent from the column was then mixed with liquid scintillation cocktail (Uniscint BD, National Diagnostics) with a flow rate of 5 mL/min. Radioactivity was monitored continuously with a Flo-One radioactivity detector (Packard Instruments, Downers Grove, IL). The analysis system was automated by use of a Waters 717 autosampler (Milford, MA). The retention times of all components were measured and recorded as follows: pyruvate, 4 min; S3P, 5 min; PEP, 10 min; EPSP ketal, 13 min; EPSP, 19 min; and tetrahedral intermediate, 25 min.

SDS−PAGE Experiments. Rapid quench experiments to look for enzyme-bound covalent intermediates typically required electrophoresis of part of the quenched reaction mixture (30 μL). Glycerol (5%) and bromophenol blue (0.01%) were added to facilitate gel loading onto a 15% polyacrylamide gel (Bio-Rad) using the standard Laemmli buffer system (*22*). Quantitation of the phosphor images (using a Bio-Rad GS250 molecular imager system, Hercules, CA) of the gels allowed determination of the relative amounts of radioactivity associated with EPSP synthase (bound) and the dye front (free). Molar concentrations were then calcu-

lated on the basis of the distribution of counts arising from the concentration of [^{32}P]PEP present in the incubation mix prior to quenching. In some cases, the stock [^{32}P]PEP was found by ion-exchange HPLC to be contaminated with up to 20% [^{32}P]phosphate. Therefore, in calculations of the molar concentrations of each species, that portion of the radioactivity of [^{32}P]phosphate was assumed to have run at the dye front and was accordingly subtracted from the free and total PEP.

A control sample (enzyme quenched before substrates added) was routinely run to verify quenching and recovery of total radioactivity in both HPLC and electrophoretic analysis. During HPLC chromatography, a "cold" standard, of PEP, was included and its retention was monitored in-line by absorption at 220 nm to confirm retention times and recovery.

Data Analysis. Curves were fit to the data, from rapid quench experiments, with the kinetic simulation program KINSIM, using numerical integration, as described previously (*4*, *23*).

Centricon Analysis. Due to lack of sensitivity, the rapid quench samples, generated using [1-^{14}C]PEP, were not quantitated using electrophoresis. Aliquots of the quenched mixture (30 μL) were added to microcon concentrators (Amicon) and increased in volume to 100 μL with HEPES buffer. The samples were concentrated, and 10 μL aliquots were taken from above and below the membrane and counted by a scintillation counter to determine their radioactivity content. A further assay was performed using quenched enzyme to assess the stability of the gel membrane to quench conditions. Electrophoretic analysis of the mixture above and below the membrane showed that no protein had passed through the membrane, as a result of membrane degradation under basic conditions.

RESULTS

Rapid Chemical Quench Experiments. One criticism of our previous kinetic analysis is that we did not observe the tetrahedral intermediate directly; rather, we quenched with acid and observed the formation of pyruvate as the breakdown product from the tetrahedral intermediate. In subsequent work, we isolated the intermediate in the presence of phosphate in order to sustain the internal equilibrium (*13*), and so it was argued that species **I** was formed by reaction of EPSP with phosphate. Although at the time, having established that the tetrahedral intermediate **I** was stable under basic conditions (*14*), we performed single turnover experiments by quenching with base to show the transient formation of the tetrahedral intermediate **I** (Anderson and Johnson, unpublished results). In our present studies, as illustrated in Figure 3, we show the results of single turnover experiments performed with either ^{14}C- or ^{32}P-labeled PEP. We have also demonstrated that the tetrahedral intermediate **I** is stable under basic quench conditions (*14*). Aliquots of the quenched reaction material were analyzed by HPLC. Figure 3 shows the time dependence of the reaction along with the best fit to the reaction calculated by computer simulation using the rate constants previously published (*4*, *23*). The results showed that during early phases of the reaction the substrate decayed and the intermediate **I** was formed, reaching a maximum concentration at around 5 ms.

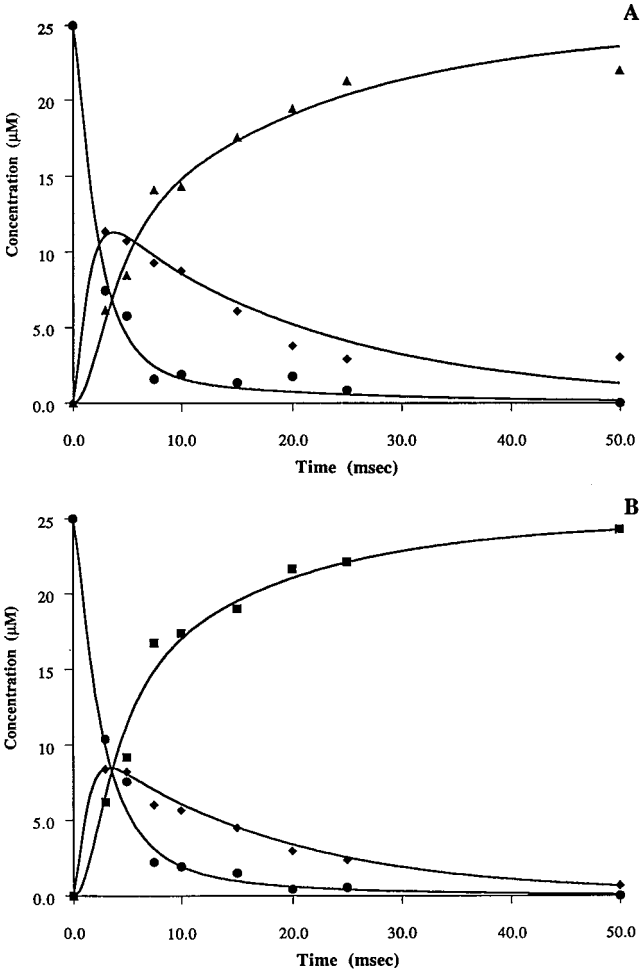

FIGURE 3: Kinetics of a single turnover experiment in EPSP synthase. (A) A solution containing enzyme (60 μM) preincubated with S3P (200 μM) was mixed with [1-^{14}C]PEP (●) (25 μM) at 20 °C. (B) A solution containing enzyme (60 μM) preincubated with S3P (200 μM) was mixed with [^{32}P]PEP (●) (25 μM) at 20 °C. The reaction was terminated by quenching with 0.2 N KOH (all concentrations cited are final concentrations after mixing). The disappearance of substrate (●), formation and decay of intermediate (◆), and formation of product, EPSP (▲) or P_i (■), were monitored by HPLC with on-line radioactivity detection. The curves shown represent a fit to the data using numerical integration with KINSIM according to the rate constants previously published (*4*).

Product was observed to form, either directly as [^{14}C]EPSP (Figure 3A) or indirectly by formation of [^{32}P]phosphate (Figure 3B). All of the radiolabel in each sample was accounted for in the sum of species observed, indicating that the labeled substrate reacted only to form the identified soluble species and no radiolabel was lost as might be expected if a covalent enzyme intermediate had formed.

Prospecting for Covalently Bound Enzyme Species. To look further for possible covalent enzyme-bound species, we followed a procedure that led to the identification of covalent species with the enzyme *MurZ* (*10*, *11*). We have previously established that covalent enzyme species such as the enzyme−phospholactoyl species **A** shown in Figure 2 are stable under basic conditions (*10*). According to the alternate mechanism proposed by Schaefer, the formation of the enzyme−phospholactoyl **A** is a prerequisite for subsequent formation of the enzyme−enolpyruvate species **B** and enzyme−ketal species **C**. Accordingly, if this mechanism were correct, we might predict that we could observe the

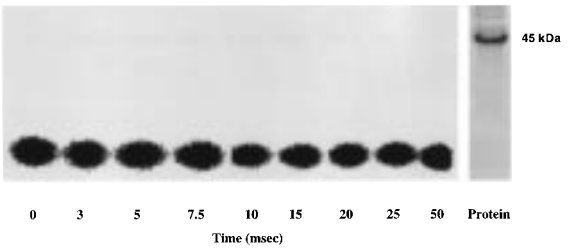

FIGURE 4: Gel analysis of a single turnover experiment in EPSP synthase: looking for covalent enzyme intermediates (**A**, **B**, or **C**; Figure 2). A solution containing enzyme (60 μM) preincubated with S3P (200 μM) was mixed with [^{32}P]PEP (25 μM) at 20 °C. The reaction was terminated by quenching with 0.2 N KOH (final concentration). The polyacrylamide gel shows radiolabeled components and protein, quenched at various time points during the single turnover reaction. No radiolabel is associated with the protein at any time point.

enzyme−phospholactoyl species **A** in single enzyme turnover experiments using [^{32}P]PEP as a substrate coupled with SDS−PAGE analysis. Thus, if **A** accumulates to a level sufficient to allow detection according to the reaction kinetics, using [^{32}P]PEP with a very high specific activity, we could detect the enzyme−phospholactoyl species **A** even if it only represented 0.1% of the total amount of radioactivity. Therefore, in examining the enzymatic reaction under single turnover conditions using [^{32}P]PEP, aliquots of the quenched reaction were mixed with glycerol and bromophenol blue and loaded onto an acrylamide gel (15%). The gel was quantified by using a Phosphorimager, revealing only one band migrating with the dye front. The Coomassie-stained gel identified the position of the protein. A time course for the conversion of [^{32}P]PEP to product under single turnover conditions is shown in Figure 4 after SDS−PAGE/phosphorimaging analysis. *No* radiolabeled bands were observed to migrate with the protein. This indicates that no phospholactoyl covalent intermediate **A** (Figure 2) was present in the quenched protein mixture.

To identify any covalently bound intermediate species enolpyruvoyl species **B** and enzyme−ketal **C** (Figure 2) due to the interaction of PEP with the enzyme, aliquots of the [^{14}C]PEP-quenched reaction mixture from the single turnover experiments were analyzed. The aliquots were concentrated using Centricon concentrators. Aliquots of the mixtures above and below the concentration membrane were taken, and the radioactivity content was determined by scintillation counting. Since the intermediate **I** was observed by HPLC to be present at a maximum concentration at around 5 ms, it is reasonable to suggest that any covalently bound intermediates, **B** and **C**, directly involved with the formation of the tetrahedral intermediate **I** would also be present during this time period. No difference in the concentration of radiolabel was observed between the mixture above and below the Centricon membrane. This indicates that *no* covalent interaction could be detected between the enzyme and any ^{14}C label.

Examination of the Reaction under Conditions for Preparing Solid-State NMR Samples. The reaction conditions described in preparation for the solid-state NMR experiments were repeated, and at various times, samples were quenched and analyzed by HPLC, using [1-^{14}C]PEP. The methanolic enzyme solutions were prepared and cooled to −30 °C. The substrates were then added. The mixtures were then quenched

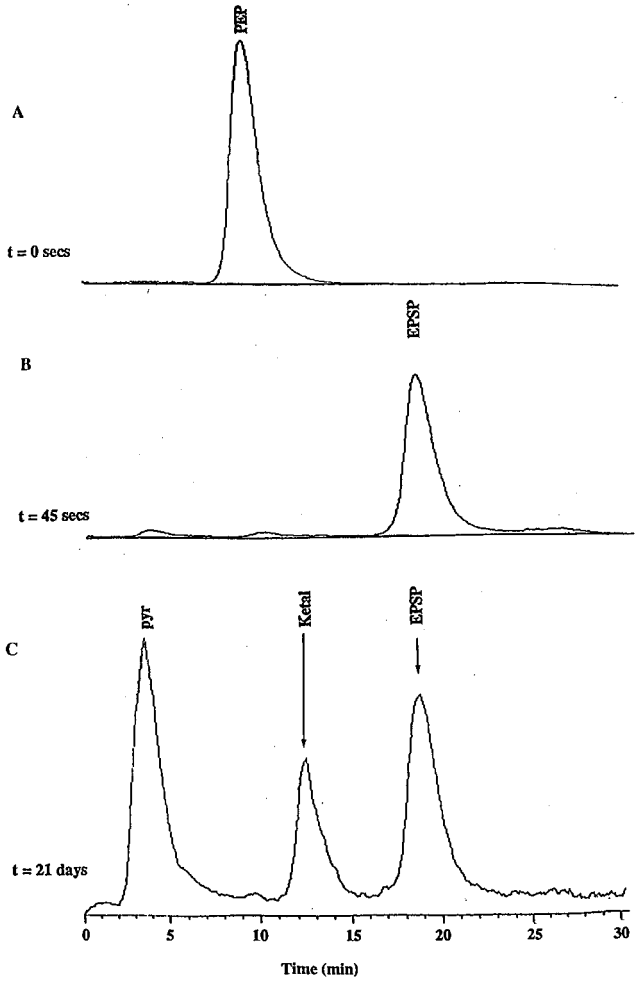

FIGURE 5: HPLC analysis of reaction catalyzed at −30 °C. A solution containing enzyme (75 μM) was mixed with methanol (to 35%) on ice and then cooled to −30 °C. The substrates (S3P and [1-^{14}C]PEP; 75 μM each) were dissolved in methanol, cooled to −30 °C, and then added to the enzyme solution. The solutions were quenched with 0.2 N KOH at (A) 0 and (B) 45 s (final concentrations). The aliquots were then analyzed by HPLC. HPLC analysis of reaction catalyzed at −30 °C after 21 days. Samples were prepared as above. The samples were not quenched with base at 45 s, but were frozen in liquid nitrogen and lyophilized. The lyophilized solid was stored at −20 °C for 21 days and analyzed by HPLC (C), following resuspension in 0.2 N KOH.

by manually adding cold base after 45 s of reaction and then analyzed by HPLC. A control zero time sample was prepared by first adding base to the enzyme and then substrate. Previous observations showed that under these quench conditions the enzyme was rapidly deactivated but the substrates, tetrahedral intermediate, and products as well as the EPSP ketal were stable (*14*). The solutions incubated at −30 °C showed total conversion of substrate to product within 45 s. It was concluded that although the conditions may have reduced the enzymatic rate significantly, turnover was rapid even at −30 °C and the reaction was complete even before the sample was frozen in liquid nitrogen (Figure 5A,B). Accordingly, the fundamental assumption that the reaction was just beginning at the start of the solid-state NMR experiment was proven to be false.

Over a period of 21 days, aliquots of the lyophilized reaction mixture were analyzed at regular intervals, by HPLC. The initial aliquot showed solely the product, EPSP. The retention time of this peak was consistent with that of

an authentic sample of EPSP (*4*, *15*, *25*). Over 21 days, two further peaks were observed to form. The peak at 5 min on the chromatogram was shown to be pyruvate, and the peak at 13 min was the EPSP ketal **II** (Figure 5C) (*15*, *25*, *30*). The retention time of the new peak was consistent with that of previous studies using an authentic sample of ketal. Both peaks formed from the decay of EPSP at −20 °C.

DISCUSSION

We present unequivocal evidence to show that the methods of sample preparation for the solid-state NMR experiments by Schaefer and co-workers (*17*) were flawed in that the reaction was completed before the start of the NMR experiment. Moreover, our data as well as those of others, using an NMR strategy (*18*), indicate an error in the solid-state NMR experiment in the assignment of the putative intermediates as an enzyme−enolpyruvate species **B** and enzyme−ketal species **C**. Compelling evidence provided by kinetic experiments examining radiolabeled substrates as well as an NMR strategy strongly suggest that those species observed are actually the product of the enzymatic reaction, EPSP, and the EPSP ketal, which is a side product formed at a rate a millionfold slower than catalysis. It is unfortunate that previous information on the mechanism and kinetics of EPSP synthase that could have been used as a guide in the development of new NMR methods was largely ignored.

The design of the solid-state NMR experiments of Schaefer and co-workers depended upon two assumptions. First, it was assumed that the reaction did not occur at −30 °C prior to freezing and lyophilization. Second, it was assumed that the enzymatic reaction continued in the lyophilized solid-state. These assumptions were essential to allow the interpretation of the changes in NMR peaks, during their observed time course over the course of several days, in terms of new intermediate species appearing on the pathway to form products. We conclusively show that the reaction in the samples at −30 °C reached completion prior to being frozen and lyophilized. Moreover, we show that new species appearing at −20 °C in the lyophilized sample are the known breakdown products, pyruvate and the EPSP ketal, characterized previously. While we cannot decisively conclude that the reactions to form these products are nonenzymatic, we have previously shown that the EPSP ketal can be formed nonenzymatically from the intermediate (*15*). The formation of the ketal from EPSP in the lyophilized material may involve the formation of a protonated vinylic species; alternatively, a less plausible pathway may involve the S_N2 attack of the C4 hydroxyl on the tetrahedral center of the intermediate (Figure 6).

Although solid-state NMR can provide unique information for the measurement of distances in ligands bound to enzymes, care must be taken in sample preparation to achieve meaningful results. Most importantly, the issue of kinetic competence cannot be ignored in proposing enzyme intermediates.

There are several important criteria for the identification of an enzyme intermediate: kinetic competence and structural proof of the proposed intermediate, observation of the intermediate bound to the enzyme, complete thermodynamic analysis of the reaction pathway and rationale based upon sound chemical principles (*24*). Although not all of these

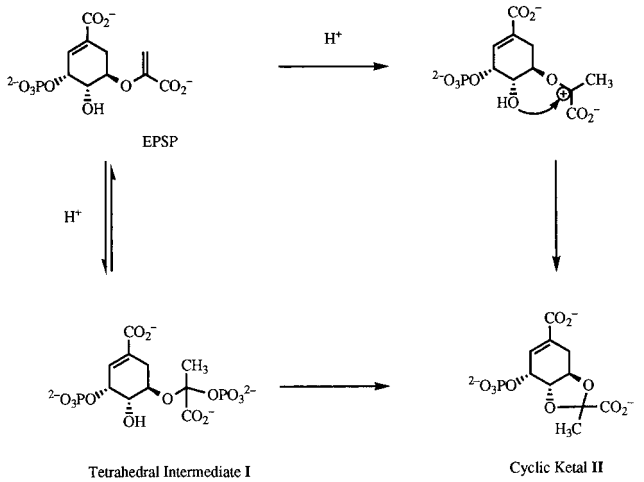

FIGURE 6: Formation of cyclic ketal **II** from EPSP or tetrahedral intermediate **I**. The observation of buildup of tetrahedral intermediate **I** could have been due to the mutation of residues preventing active site facilitated degradation of the tetrahedral intermediate **I**. Since the residues mutated do not have charged side chains, with the exception of the N94S mutation, their direct involvement in catalysis seems unlikely. However, the mutations may have altered the tertiary enzyme structure, thus rendering the catalytic residues too remote from the site of reaction to catalyze the degradation effectively, thus indirectly influencing the active site reaction. It was previously reported that the binding affinity of S3P was impaired by the presence of one of the ^{19}F-tryptophans, positioned less than 10 Å from the active site (*28*). It is therefore possible that binding of the intermediate structure **I** may also be impaired in the RC mutant.

criteria can be achieved due to technical limitations with most enzyme systems, all have been achieved in the case of EPSP synthase and the tetrahedral intermediate (*24*). It was surprising to see the results and discussion presented by Schaefer and co-workers (*17*), ignoring the significant body of prior work on EPSP synthase.

We have addressed criticisms of our previous work raised by Schaefer and co-workers by presenting new data on the time course of formation of the tetrahedral intermediate in the *absence* of added phosphate and by once again looking for species covalently bound to enzyme. Our original rapid chemical quench experiments leading to the identification of the tetrahedral intermediate were performed by quenching with acid, leading to the breakdown of the intermediate to pyruvate (*4*). Subsequent work showed that the intermediate was stable in basic solutions and that it could be isolated and added back to the enzyme, partitioning to form both substrates and products according to our previously defined kinetics (*14*). However, we had not yet published the time course of formation and decay of the tetrahedral intermediate as observed by quenching with base, preserving the integrity of the intermediate. The data presented in this paper provide this information, providing unequivocal proof for the kinetic competence of the tetrahedral intermediate. Furthermore, the tetrahedral intermediate **I** has also recently been observed in the absence of phosphate or chemical quenching agents using a novel rapid mixing, pulsed-flow/electrospray ionization mass spectrometry technique (*25*).

Identity of Species Observed by Solid-State NMR. The solid-state NMR data followed the conversion of a signal at 155 ppm to 108 ppm. The species at 155 ppm has been attributed to the covalent enolpyruvoyl species **B** (Figure 2). The species arising from the C-13 resonance at 108 ppm

contains no local C-13−phosphate coupling. It is argued that this species contains a shikimate ring that is phosphorylated only at C3 and is not the tetrahedral intermediate species **I**, but rather the previously unobserved S3P ketal species **C**. It has been suggested that species **C** is covalently bound to the enzyme and arises from species **B** according to the mechanism outlined in Figure 2. However, the results from the Centricon studies do not support these conclusions as *no* covalently bound species are observed. Moreover, the HPLC analysis implies that the reaction observed by solid-state NMR is simply the conversion of the sp^2 product EPSP to the sp^3 quaternary ketal **II** (Figure 1). Previous solution ^{13}C NMR studies have shown that the chemical shift for the quaternary carbon for EPSP is 156 ppm and the quaternary carbon for the ketal is 107 ppm (*15*). In previous analyses, the ketal **II** was reported to be very stable and formed irreversibly, presumably from the decomposition of the tetrahedral intermediate **I**. The slight difference in chemical shifts may simply be attributable to solvent effects, or lack thereof in the solid-state.

Reaction Rates at −30 °C. At first it is perhaps surprising that the reaction reached completion so rapidly at −30 °C, a fact not even considered by Schaefer and co-workers. However, closer analysis of known kinetic parameters reveals that one might have expected such a fast reaction. The reactions for both mutants and wild-type enzyme were examined with enzyme in 1:1 stoichiometry with substrates, so that the enzyme needed to only catalyze a single turnover. The half-life of the active site reaction at 20 °C is approximately 5 ms, as measured from solution single turnover experiments. Assuming that the rate decreases by a factor of 2.5 times for every 10 °C drop in temperature, this only predicts a half-life of about 200 ms, at −30 °C. Since the mutant is 30 times slower than the wild type enzyme, the half-life of the mutant reaction could be perhaps 6 s. In preparation for solid-state NMR, the reaction mixture was allowed to incubate under single turnover conditions for 45 s prior to freezing, thus allowing 7.5 half-lives to occur, which is more than sufficient to observe total turnover. Although the activity of the enzyme was not measured under the conditions used prior to lyophilization, a reasonable extrapolation from previous measurements would have revealed a flaw in the design of the experiment. However, the changes in solution conditions, the addition of cyroprotectants, disaccharides, and methanol, may clearly affect the reaction kinetics. Moreover, the change in temperature may alter the internal equilibrium governing the formation of intermediates at the active site. These changes demand a more extensive characterization of the reaction kinetics prior to and during the preparation of samples for solid-state NMR.

Scrambling of the Vinylic Protons of PEP. Other evidence was cited by Schaefer and co-workers in support of an enzyme-bound nucleophile. This speculation was initially raised by investigations carried out by Anton (*7*) based upon the observation that in the absence of the C4 or C5 hydroxyl of S3P, PEP was shown to still exchange vinylic protons with tritium-labeled water, a reaction occurring during normal turnover of S3P to EPSP (*4*). It was therefore proposed that the C5 hydroxyl did not serve as the nucleophile in the reaction, implying the need for an enzymic nucleophile to account for the observed exchange. However, an explanation for Anton's observation has been put forth by previous work in this laboratory based upon a knowledge of the reaction pathway and kinetics (*4*). The pathway of formation of the tetrahedral intermediate from S3P and PEP implies the involvement of acidic and basic residues to protonate the vinylic sp^2 protons on the C3 of PEP and deprotonate the C5 hydroxyl of S3P, respectively. The rate of exchange in the presence of dideoxy S3P or C5 deoxy S3P was approximately 800 times slower than the rate of formation of the tetrahedral intermediate from S3P (*16*, *26*). This slow exchange reaction can be accounted for by proposing the formation of an unstable carbocation, stabilized by active site residues normally involved in the formation of a tetrahedral intermediate. One might expect such a side reaction to occur on a time scale far slower than the normal reaction. Thus, one can account for the slow exchange reaction observed with artificial substrates lacking the C5 hydroxyl without the need to invoke new enzyme intermediates for which there is no supporting data.

Solid-State NMR Studies on the RC Mutant Experiment. In the studies using the RC mutant, Schaefer and co-workers (*17*) observed the formation of the tetrahedral intermediate **I**. However, they proposed that it was generated by the reaction of EPSP and P_i, binding to active enzyme in the solid-state, rather than simply as an intermediate formed upon reaction of S3P and PEP. The activity of the RC mutant was only determined, at 30 °C under steady-state conditions, by the release of phosphate. From this it was deduced that EPSP was forming and hence the enzyme was catalytically active. However, an alternate explanation is that the mutant enzyme may have catalyzed the formation of the intermediate, but was impaired in catalyzing the breakdown of the intermediate to form products. Any intermediate formed during the reaction would have broken down nonenzymatically to S3P, pyruvate, and phosphate under the acidic conditions of the phosphate assay (*14*, *27*).

Previous studies of the reaction kinetics for the wild-type enzyme have shown that there is an internal equilibrium at the active site between substrate, the tetrahedral intermediate, and product such that 30% of the enzyme active sites are occupied by the intermediate (*4*). If indeed the breakdown of the intermediate were impaired in the mutant enzyme, one would predict a larger buildup of this species. It is interesting to speculate the features of the reaction kinetics that may be responsible for the larger buildup of tetrahedral intermediate in the mutant enzyme as compared with wild type. One might predict that these mutations result in an impaired rate of breakdown of the intermediate, and therefore an increased level of the tetrahedral species **I** would be observed. This is supported by the ^{31}P NMR experiments with the mutant enzyme. In these experiments, the dephased ^{31}P NMR signal intensity, identified as the P−C coupling at the tetrahedral center, was equivalent to the dephased signal from the C3 phosphate, suggesting that only one species, **I**, has accumulated at the active site.

Clearly a more detailed kinetic analysis of the reaction pathway for the mutant enzyme is required to more fully explore the nature of the defect in catalysis.

NMR Analysis of Double Mutant and Wild-Type Enzyme. The results obtained using the double mutant and wild-type enzyme were used to suggest a series of enzyme-bound intermediate species **A**, **B**, and **C** (Figure 2). The phosphate decoupling experiments suggested that the "enzyme bound

species" **B** and **C** did not contain phosphate, other than at C3 of S3P. Since only product was bound to the enzyme, we have shown that these observations are attributable to the decomposition of product to cyclic ketal **II**. Both EPSP and cyclic ketal **II** were observed by HPLC. The ketal could be generated either via the tetrahedral intermediate **I** following rephosphorylation of EPSP or by direct protonation of the vinylic sp^2 carbon of EPSP followed by cyclization (Figure 6). The lack of observation of the tetrahedral intermediate **I** suggests that the latter case is more likely. The formation of ketal **II** from EPSP would also explain the observed decrease in the measured distance between the phosphate of S3P and the carbon centers from 7.5 to 6.1 Å.

Conclusions. We have provided evidence to show that the transformations observed by solid-state NMR can be accounted for by degradation of the enzyme product EPSP, present at the start of the experiments. It is clear that solid-state NMR methods can play an important role in identifying enzyme intermediates (*28*, *29*). However, to provide meaningful conclusions regarding enzyme mechanism, these data must be interpreted in the context of the available biochemical information emphasizing important criteria for establishing true reaction intermediates such as kinetic competence.

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