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Slowed Enzymatic Turnover Allows Characterization of Intermediates by Solid-State NMR†

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ABSTRACT: EPSP (5-enolpyruvylshikimate-3-phosphate) synthase catalyzes condensation of shikimate 3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form EPSP, a precursor to the aromatic amino acids. S3P and [2-^{13}C]PEP were bound to mutant or wild type *E. coli* forms of the enzyme prior to lyophilization. CPMAS-echo and rotational-echo double-resonance (REDOR) NMR experiments, employing a slow catalytic EPSP synthase mutant and a long prelyophilization incubation interval, allowed our observation of the gradual formation of a strong ^{31}P−^{13}C coupling consistent with the well characterized tetrahedral intermediate. However, after shorter low temperature incubation intervals of substrates with mutant or wild-type enzymes, carbon CPMAS-echo NMR spectra showed the ^{13}C label at 155 ppm, consistent with sp^2 geometry of this carbon. REDOR revealed that the phosphorus of PEP was cleaved. However, phosphorus at a distance of 7.5 Å was observed, due to the phosphate of a nearby bound S3P. Heating the sample allowed the reaction to progress, as shown by the diminution of the 155 ppm peak and growth of a peak at 108 ppm. The sp^3 geometry implied by the 108 ppm peak strongly suggested formation of a S3P−PEP condensation product. REDOR indicated that phosphorus was still distant, but now only 6.1 (wild type) or 5.9 Å (mutant) distant. We think that the early intermediates with peaks at 155 and 108 ppm are covalently bound to the enzyme. We also think that the tetrahedral intermediate that we observed was formed after product was generated.

We describe a new and potentially general method of determining the pathway of an enzymatic reaction via solid-state magic-angle spinning (MAS) NMR.[1] We have discovered that sub-zero substrate entrapment in EPSP synthase followed by lyophilization in the presence of stabilizing protectants results in ultraslow catalysis in the solid-state. This facilitated the discovery of previously uncharacterized intermediates proximal and distal to the catalyzed condensation of the substrates. Cooling the sample temporarily halted the reaction, allowing structural information about each intermediate to be gathered by REDOR *(1,2)*, a well-

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[1] Abbreviations: NMR, nuclear magnetic resonance; EPSP, 5-enolpyruvylshikimate-3-phosphate; S3P, shikimate 3-phosphate; PEP, phosphoenolpyruvate; CPMAS, cross polarization magic angle spinning; REDOR, rotational echo double resonance; ppm, parts per million; MAS, magic angle spinning; PCR, polymerase chain reaction; PEG, polyethylene glycol; MW, molecular weight; DTE, dithioerythritol; MOPS, 3-morpholinopropanesulfonic acid; HEPES, *N*-(2-Hydroxyethyl)piperazine-*N*′-ethane-2-sulfonic acid; CP, cross polarization; OD, outer diameter; RC, recombinant circle; RF, radiofrequency; N_c, number of rotor cycles; P_i, inorganic phosphate; UDP, uridine 5′-diphosphate; ΔS, REDOR difference; S_o, full echo.

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characterized and reliable *(3−5)* method of obtaining accurate *(6,7)* heteronuclear distances.

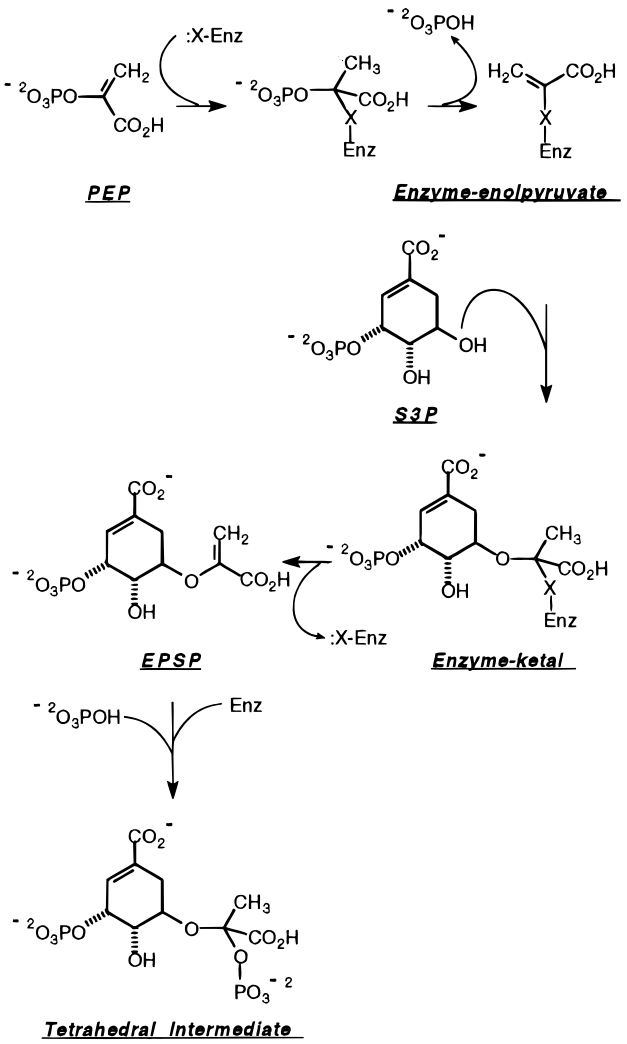
EPSP synthase is an enzyme restricted to plants and microorganisms. It catalyzes the condensation of shikimate 3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form inorganic phosphate (P_i) and EPSP, a precursor to the aromatic amino acids. This reaction is inhibited by *N*-(phosphonomethyl)glycine (glyphosate), which forms a stable ternary complex with the enzyme and S3P. This fact has been exploited in the use of glyphosate as a herbicide nontoxic to mammals. As a consequence, EPSP synthase has been the subject of intensive research.

A crystal structure suggests the EPSP synthase active site is in a cleft between two globular domains *(8)*. We have employed solid-state MAS NMR spectroscopy to map the structure of the ternary glyphosate complex, which resides in this cleft *(9−11)*. As part of this effort, mutant enzymes were created (W289Q and F172W, W289Q: referred to as single and double mutants), by manipulating the positions of the wild-type tryptophans. These were used to obtain long-distance REDOR constraints between the globular domains using the high gyromagnetic ratio provided by incorporated [6-^{19}F]tryptophan *(11)*. The current studies began with a slow catalytic mutant (N94S, I113M, F172W, W289Q; or RC mutant) first described in this paper. As our work with EPSP synthase reaction pathways in solid-state reactions progressed, we switched to the less perturbed double mutant and, finally, the wild-type enzyme.

The mechanism originally proposed *(12)*, and currently accepted *(13)*, is that the enzyme catalyzes the addition of S3P(C5)-OH to the double bond of PEP to form a tetrahedral intermediate (so-called because PEP C2 is converted from sp^2 to sp^3 hybridization). This is followed by the elimination of the enolpyruvoyl phosphate to form EPSP. However, an alternate mechanism was advanced by Anton et al. *(14)*. They found that tritium was incorporated into PEP from the solvent in the presence of an S3P analogue that lacked the C-5 OH group. This indicated that an enzyme nucleophile was involved in catalyzing the formation of a methyl from the β-carbon methylene of PEP, allowing the exchange of solvent protons into this moiety. They suggested that a negatively charged active-site residue stabilized a PEP α-carbonium ion and/or formed a covalent bond to the α-carbon of PEP. S3P could attack to form the tetrahedral intermediate. Conversely, phosphate could be eliminated to form a covalent enolpyruvyl−enzyme adduct which would react with S3P to form EPSP.

We believe that the latter scheme advanced by Anton et al. *(14)* is correct. Our observation of the forward reaction strongly supports the formation of an enzyme−PEP complex, followed by elimination of the phosphate to form a covalent enolpyruvyl−enzyme adduct. However, when S3P-OH adds to the double bond of this adduct, a third intermediate is formed, a covalently bound condensation product (an enzyme−ketal), which subsequently undergoes cleavage to form EPSP (Scheme 1).

Scheme 1



EXPERIMENTAL PROCEDURES

Enzyme Production and Purification. The plasmid pMON5537 (a gift of Monsanto company, St. Louis, MO), which contains the coding region for wild-type EPSP synthase is described elsewhere *(10)*. Site-directed mutagenesis was accomplished by PCR. The single mutant (pEPSPSW289Q) was produced using primers containing unique restriction sites, one of which was mutagenic. The RC mutant (pEPSPSRC) was produced using pEPSPSW289Q as a template in a "reverse circle" method wherein the entire plasmid is amplified *(15)*. The double mutant (pEPSPSOI) was produced using PCR primers plus a phosphorylated mutagenic oligonucleotide incorporated during amplification to effect the F172W change *(16)*.

All enzymes contained biosynthetically incorporated [6-^{19}F]-tryptophan. The method for bacterial overexpression, employing an *E. coli* strain auxotrophic for tryptophan, W3110trp A33, is similar to that detailed in McDowell et al. *(10)*. The only difference is that NH_4Cl (1 mg/mL) was used instead of [^{15}N]-labeled $(NH_4)_2SO_4$, and amino acids at 100 mg/mL were provided in both initial and final media.

Enzyme purification was as described *(10)*, except that after concentration of the final Q-sepharose column fractions by ultrafiltration, buffer exchange was effected by several 60 to 6 mL concentration steps with 1 mM dithioerythritol (DTE), 2 mM MOPS, pH 7.2. PEG 8000, dissolved in the same buffer, was added for a 1% (w/v) final concentration before storage at −80 °C.

Enzyme Kinetics. S3P kinetic constants for the RC mutant of EPSP synthase were obtained using the phosphate release assay as employed by Studelska et al. *(11)*. As was the case

for the double mutant (F172W, W289Q), the enzyme concentration was 98.5 nM in 200 mM HEPES/tetramethylammonium hydroxide buffer, pH 7.0. The reaction time was 5 min at 30 °C. S3P concentrations ranged between 1.5 and 192 μM, and the PEP concentration was set at 96 μM.

Sample Preparation. The RC mutant was lyophilized at 65 μM with S3P and [2-^{13}C]PEP at 65 μM in 20 mM sucrose, 1% (w/v) PEG 8000 (mean MW), 1 mM DTE, 2 mM MOPS, pH 7.2. The double mutant was lyophilized at 175 μM with S3P and [2-^{13}C]PEP at 175 μM in 20 mM lactose, 0.85% (w/v) PEG 8000, 1 mM DTE, 2 mM MOPS, pH 7.2. For substrate entrapment in wild-type EPSP synthase, 100 mg of wild type *E. coli* EPSP synthase was dissolved in 21 mL of 20 mM trehalose, 1% PEG 8000, 0.7 mM DTE, 1.4 mM MOPS, pH 7.2. While stirring on ice, 11 mL of cold methanol was added dropwise to the lyophilization flask (final concentration 35% v/v). The solution was cooled to −30 °C, and then S3P and [2-^{13}C]PEP, in 220 μL of cold 50% methanol, were added while swirling the flask. The final enzyme concentration was 74 μM and each substrate was 98.6 μM. After 45 s, the solution was frozen by placing the flask in liquid nitrogen. The flask was attached to a lyophilizer with a −115 °C cold trap, and a 5 mTorr vacuum was established before the flask was transferred from liquid nitrogen to a regulated −80 °C bath. Sublimation was monitored by use of a vacuum sensor mounted close to the flask. Lyophilization was accomplished by gradually increasing the temperature of the bath toward room temperature.

Lactose or trehalose were employed in later experiments instead of sucrose because their carbons are all protonated. This allowed the removal of the natural-abundance ^{13}C peaks of disaccharide and PEG from the carbon NMR spectra by inserting a delay in proton decoupling *(18)*. Trehalose was later preferred over lactose, a reducing sugar, to avoid the potential problem of protein browning.

NMR. Instruments with 4-channel probes and ^{1}H Larmor frequencies of 200 MHz *(17)* or 300 MHz *(10)* were employed with a stack-mounted cooling system *(17)*. MAS employed 7.5 mm (OD) rotors at 4167 Hz for carbon-observe, and 5000 Hz for phosphorus-observe experiments. RF field strengths were 38 or 50 kHz for CP and dephasing pulses, and 90 kHz for proton decoupling. For carbon spectra, a decoupling delay of 50−60 μs eliminated signals from carbons directly bonded to protons *(18)*. The CP time was 1 or 2 ms, and the experiment repeat time was 2 s.

RESULTS AND DISCUSSION

RC Mutant. An early version of a double-mutant EPSP synthase, that had the intended W289Q and F172W changes, with [6-^{19}F]tryptophans incorporated, but also unintended changes (N94S, I113M), exhibited slow catalysis. Kinetic parameters for S3P in the forward direction determined for this mutant (hereafter called the RC mutant), were (mean ± standard deviation): $K_{m,apparent} = 2.4 \pm 0.3$ μM and $V_{max,apparent} = 0.9 \pm 0.1$ μM P_i min^{-1}. This is 90 times slower than the wild-type enzyme and 3 times slower than the double mutant *(11)*.

This slow catalytic rate prompted us to prepare a lyophilized complex with the natural substrates bound to the RC mutant at a 1:1:1 ratio. Our first experiment (results not shown) used 45 mg of the RC mutant, which was frozen and lyophilized after a 30 min room temperature incubation with S3P and PEP. Phosphorus CP-echo NMR revealed a sharp peak with the same chemical shift as S3P phosphorus. We were amazed to see the intensity of this peak subside as a new downfield peak appeared over 4 days of room-temperature data collection. These changes suggested that the condensation reaction catalyzed by EPSP synthase was progressing in the solid-state.

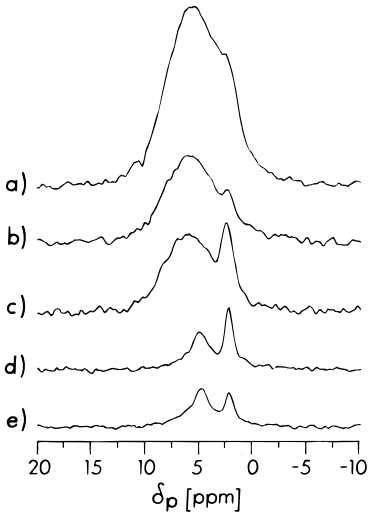


FIGURE 1: Sequential 121-MHz ^{31}P CPMAS-echo NMR spectra of RC mutant EPSP synthase complexed with S3P and [2-^{13}C]PEP. Room temperature operation allowed catalysis to proceed. Because of <0 °C interruptions for CP-echo and REDOR experiments, the reaction took several weeks. Spectra were normalized with respect to scans: (a) 40960, (b) 26784, (c) 8192, (d) 33088, and (e) 40960 scans.

In a second experiment, 90 mg of the RC mutant was complexed with substrates at 1:1:1. S3P was added first, for a 30 min incubation on ice, and then [2-^{13}C]PEP was added for an additional 30 min before freezing and lyophilization. The initial phosphorus CP-echo NMR spectrum showed a single broad peak, consistent with the heterogeneous immobilization of phosphorus-containing species. Because this spectrum was different from those obtained from the sample incubated at room temperature, we decided to rehydrate the sample on ice and incubate for 10 min at room temperature before refreezing and relyophilization. This time the initial phosphorus CP-echo NMR spectrum was broad, but showed evidence of an upfield shoulder (Figure 1a). After several hours at room temperature, we began to see the emergence of an upfield peak with the same chemical shift as S3P phosphorus (Figure 1b). We cooled the sample to −8 °C (temperature of exit gas from spinner head assembly) for phosphorus-observed, carbon-dephased REDOR ($N_c = 32$) and obtained a small difference signal (not shown) that indicated the presence of a two-bond ^{31}P−^{13}C coupling. The sample was again returned to room temperature until the upfield peak had grown in intensity (Figure 1c). REDOR experiments at −8 °C revealed a coupling between the sharp phosphorus peak and the labeled carbon consistent with the two-bond distance that is present in the tightly bound tetrahedral intermediate identified by Anderson et al. *(19)*. Carbon-observed, phosphorus dephased REDOR ($N_c = 16$) produced complete dephasing of a peak at 102 ppm, a chemical shift consistent with sp^3 hybridization of the ^{13}C-labeled carbon (not shown). Phosphorus-observed, carbon-dephased REDOR ($N_c = 32$) produced 50% dephas-

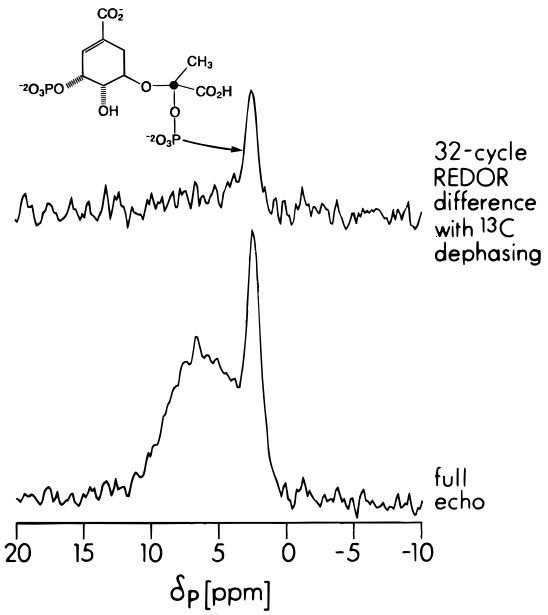


FIGURE 2: 121-MHz REDOR ^{31}P NMR spectra of RC mutant EPSP synthase complexed with S3P and [2-^{13}C]PEP after 32 rotor cycles. *REDOR difference*: Full echo spectrum minus spectrum with ^{13}C dephasing. *Full echo*: Without ^{13}C dephasing, 76176 scans. At N_c = 32, the signal from a phosphorus two bonds from an [8-^{13}C] label (●) in the tetrahedral intermediate would be completely dephased.

ing of the sharp phosphorus peak (Figure 2). Under these conditions, the signal from the phosphorus two bonds away from the labeled carbon is completely dephased. The 50% not dephased is due to the phosphate moiety on C3 of the shikimate ring. This phosphorus is six bonds away from the ^{13}C-labeled carbon, too distant to contribute to the dephasing.

Hahn-echo experiments *(20,21)* revealed that decreases in the phosphate signal during the reaction (Figure 1) were due to inefficient cross polarization caused by increased motion of phosphorus-containing molecules (spectra not shown). Mobility may be due to the cleavage and/or release of phosphate and the appearance of other species (i.e., EPSP) which have low affinity for the enzyme.

Double Mutant and Wild-Type EPSP Synthase. Subsequent experiments were performed with either the W289Q, F172W double mutant *(11)* or the wild-type enzyme. A 5 min ice temperature incubation of S3P and [2-^{13}C]PEP at a 1:1:1 ratio with 173 mg of the W289Q, F172W double mutant resulted in the entrapment of an early intermediate of the forward reaction. This intermediate suggested the formation of a covalent enzyme−enolpyruvyl adduct (see below). This finding is not consistent with the currently accepted mechanism. The catalytic rate of this enzyme was only 30-fold slower than the wild type rate *(11)*, but we were concerned about the possibility that the mutation may have altered the catalytic mechanism. Because of this concern and our desire to extend the generality of a method that appeared to depend on a fortuitous mutation, we endeavored to entrap S3P and PEP in the wild-type enzyme to see if the same early intermediates could be observed.

We employed high molecular weight polyethylene glycol (PEG), thought to act as a cryoprotectant by its exclusion from the surface of a solvated protein *(22)* and disaccharide, thought to protect a protein from damage during drying by forming hydrogen bonds *(23)* with the protein. These

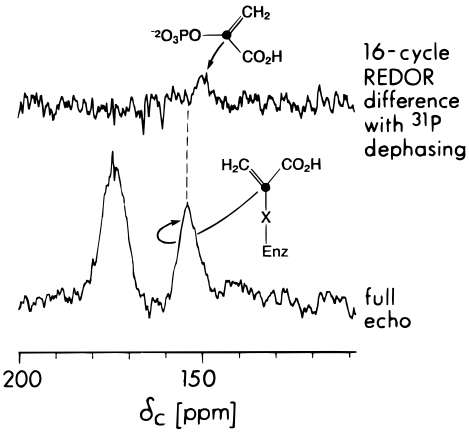


FIGURE 3: 50-MHz REDOR ^{13}C NMR spectra of wild-type EPSP synthase complexed with S3P and [2-^{13}C]PEP after 16 rotor cycles. *REDOR difference*: Full echo spectrum minus spectrum with ^{31}P dephasing. *Full echo*: Without ^{31}P dephasing, 40960 scans. There is no difference signal at 155 ppm, indicating the labeled carbon (●) is not close to a phosphorus.

measures maintain the native geometry of lyophilized proteins *(23)*. Employing these protectants, we have substantially narrowed the NMR line-widths of proteins *(Studelska, unpublished observations)* and peptides *(24)*. Optimization of cryo- and lyoprotection will require correlation of solid-state enzyme structure *(23)* and reaction rate with the water content and glass transition temperature of the sample.

Entrapped Substrates and Intermediates. To entrap substrates in the wild-type enzyme we added them after the enzyme was at −30 °C. The protein solution containing buffer, PEG 8000, and disaccharide had a freezing point of −7 °C. Because increasing the amount of these stabilizers to effect further freezing point depression would have added to the solid bulk and limited the amount of enzyme we could pack in the rotor, we added a volatile organic solvent for its antifreeze effect. We employed methanol at 35% (v/v) because of its established use as a nondenaturing protein precipitant during the early stages of protein purification *(25)*. The goal was to lower the freezing point of the solution while avoiding protein aggregation. PEG 8000 was deliberately retained in this system to function as a cryoprotectant during freezing and to prevent cold denaturation while the enzyme was in the liquid state at subzero temperatures prior to freezing.

The intermediates generated in the EPSP synthase double mutant were also observed in the lyophilized wild-type enzyme after subzero substrate addition. The reaction pathway in these two enzymes is virtually identical. In the carbon CP-echo NMR spectrum, we found evidence for a species with sp^2 hybridization of the [2-^{13}C] label of PEP. There was some intact PEP remaining on the upfield side of this peak at 150 ppm, readily detected by carbon-observed, phosphorus-dephased REDOR, but most of the signal, shifted to 155 ppm, showed no evidence of a strongly coupled phosphorus (Figure 3). With heating, this species disappeared in both samples as another species emerged at 108 ppm (Figure 4). The spectra in Figure 4b,c (at 30 °C) are less intense than 4a,d (at −6 or −5 °C) due to less efficient CP while heating and fewer signals averaged during changes induced by the reaction. Although the 108 ppm chemical shift indicated tetrahedral geometry, REDOR showed no strongly coupled phosphorus (Figure 5a). Moreover, even

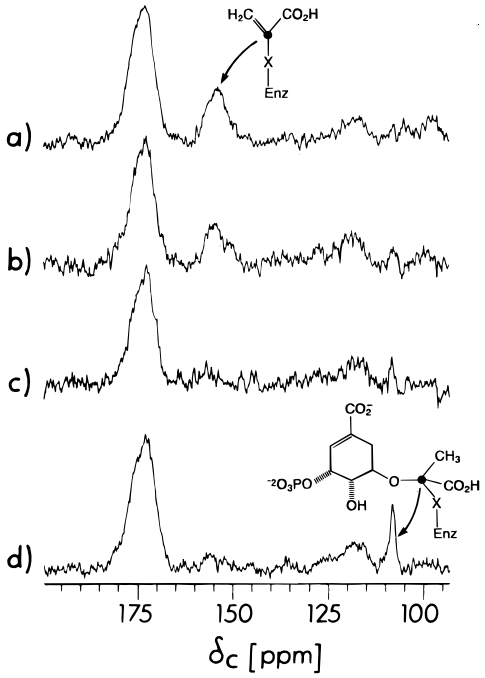


FIGURE 4: Sequential 75-MHz ^{13}C CPMAS-echo NMR spectra of wild type EPSP synthase complexed with S3P and [2-^{13}C]PEP. Changes occurred during 2 days of heating at 30 °C. (a) After the REDOR experiment shown in Figure 3. Exit gas −6 °C, 24576 scans. (b) and (c) Exit gas 30 °C, 10240 scans. (d) Exit gas −5 °C, 25600 scans. The disappearance of the 155-ppm peak and the growth of a peak at 108 ppm indicates the ^{13}C-labeled carbon (●) has changed from sp^2 to sp^3 hybridization. We propose that S3P condenses with an enzyme enolpyruvate to form an enzyme ketal.

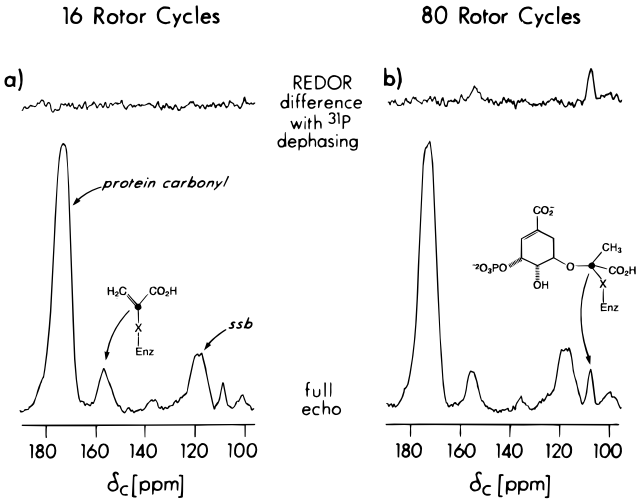


FIGURE 5: 75-MHz REDOR ^{13}C NMR spectra of wild-type EPSP synthase complexed with S3P and [2-^{13}C]PEP after 16 (left, 81920 scans) or 80 (right, 163840 scans) rotor cycles. In this sample, the reaction was allowed to progress until the difference signal at 150 ppm due to uncleaved [2-^{13}C]PEP had completely disappeared. *REDOR difference*: Full echo spectrum minus spectrum with ^{31}P dephasing. *Full echo*: Without ^{31}P dephasing. No difference signal at $N_c = 16$ for the [^{13}C]-labeled carbon (●) peaks at 155 and 108 ppm shows that phosphorus is not close to either species. The larger difference signal for the 108-ppm peak vs the 15-ppm peak at $N_c = 80$ indicates that S3P phosphorus is closer to the 108-ppm carbon.

after the sample heating that produced this change in the carbon spectrum, the phosphorus spectrum remained broad (not shown) and showed no evidence of the development of the sharp upfield line that we had found was associated with the appearance of the tetrahedral intermediate (Figure 2).

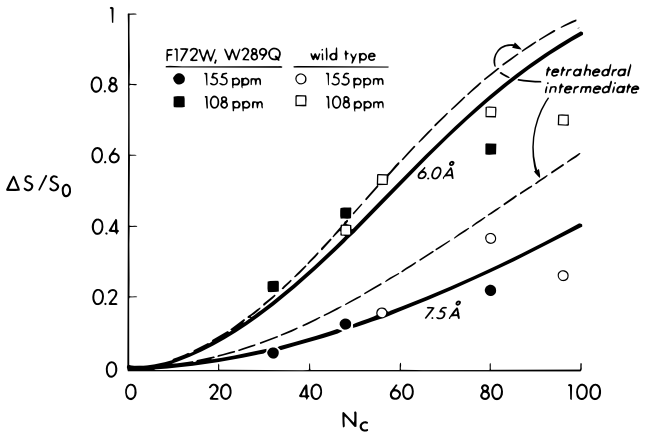


FIGURE 6: ^{13}C REDOR ($\Delta S/S_o$), with dephasing by ^{31}P for double mutant and wild-type EPSP synthase complexes. The distant phosphorus from S3P dephases the ^{13}C signal from [2-^{13}C]PEP. The solid lines indicate that S3P phosphorus is, on average, 1.5 Å closer to the ^{13}C-carbon in the 108-ppm species. The dashed lines show ± one standard deviation about the mean of allowed *intra*molecular distances.

We believe that we have trapped early intermediates preceding the well-characterized tetrahedral intermediate. In this view, the peak at 155 ppm is due to an attack by an enzyme nucleophile on PEP, resulting in the loss of phosphate to produce a covalently bound species with sp^2 hybridization of the labeled carbon. The peak which appears subsequently at 108 ppm results from the condensation of this species with S3P, to form a covalently bound enzyme−ketal with sp^3 hybridization of the labeled carbon. A precedent for a covalent PEP adduct as an intermediate can be found in UDP−*N*-acetylglucosamine enolpyruvoyl transferase, the only other known PEP transferase, which has considerable homology to EPSP synthase *(26)*. To test the hypothesis that the shift from 155 to 108 ppm resulted from condensation, we performed carbon-observed, phosphorus-dephased REDOR employing enough rotor cycles of dipolar evolution to measure the coupling between the distant phosphorus from S3P to the labeled carbon from [2-^{13}C]-PEP on samples exhibiting the early sp^2 intermediate at 155 ppm and/or the sp^3 intermediate at 108 ppm. We obtained a ^{31}P to ^{13}C distance of 7.6 ± 0.3 Å (double mutant) and 7.5 ± 0.5 Å (wild type) for the species with a 155 ppm chemical shift, and a distance of 5.9 ± 0.4 Å (double mutant) and 6.1 ± 0.3 Å (wild type) for the species with a 108 ppm chemical shift (Figure 6). Spectra for the wild-type sample are shown in Figure 5. The change in the phosphorus−carbon distance for the double mutant and wild-type samples was 1.7 and 1.4 Å, respectively, and represents a closing consistent with a condensation event.

The weak coupling of the carbon at 155 ppm to the phosphorous is quite unlikely for an intramolecular distance. The 147 unique conformers of the *R*-tetrahedral intermediate have an average ^{31}P (from S3P) to ^{13}C (from [2-^{13}C]PEP) distance (mean ± standard deviation) of 6.365 ± 0.505 Å *(9)*. This range is represented by dashed lines in Figure 6. Notice that the 108 ppm data is within this range, while the 155 ppm data is outside it, toward longer distances. In fact, only 1 of the 147 conformers had a distance of 7.5 Å or more, and its strain energy was more than 6 kcal/mol above that of the minimum energy conformer. Therefore, the long 7.5 Å ^{31}P to ^{13}C distance we observed for the 155 ppm peak strengthens the case for an *intermolecular* coupling from S3P

to a covalently bound enolpyruvyl−enzyme species. Our findings are consistent with a *short inter*molecular distance that enables a condensation reaction which results in a *shorter intra*molecular distance.

Tetrahedral Intermediate. We think it is likely that the enzyme−ketal forms product directly by rearrangement. But what about the classic tetrahedral intermediate? The case for this pathway, wherein the enzyme is thought to catalyze the direct addition of S3P to PEP to form the tetrahedral intermediate, followed by the elimination of phosphate to form product, depended on the successful isolation of this intermediate. Acid quenching used for the rapidly stopped single-turnover experiments generated pyruvate, a decomposition product that could have been derived from a covalently bound enzyme−enolpyruvate intermediate, as well as from the proposed tetrahedral intermediate *(13)*. The tetrahedral intermediate was finally successfully isolated by denaturing EPSP synthase by quenching with triethylamine at a final pH of 12 after an "internal equilibrium" had been set up with a limiting amount of S3P, but with enzyme, PEP, and phosphate in excess *(13,19)*. On the basis of our observation of the formation of the tetrahedral intermediate in early experiments with the RC mutant, we believe that it may be formed only after product is generated. We are beginning experiments to monitor the reverse reaction with labeled EPSP, taking advantage of the slow catalytic rate afforded by a lyophilized enzyme. This continuing re-evaluation of the mode of action of EPSP synthase will help determine the future role of our solid-state NMR approach for the analysis of enzymatic reaction pathways.

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