Site-Directed Mutagenesis of Putative Active Site Residues of 5-Enolpyruvylshikimate-3-phosphate Synthase†

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ABSTRACT: The site-directed mutagenesis of a number of proposed active site residues of 5-enolpyruvyl shikimate-3-phosphate (EPSP) synthase is reported. Several of these mutations resulted in complete loss of enzyme activity indicating that these residues are probably involved with catalysis, notably K22R, K411R, D384A, R27A, R100A, and D242A. Of those, K22R, R27A, and D384A did not bind either the substrate shikimate-3-phosphate (S3P) or glyphosate (GLP). The K411R and D242A mutants bind S3P only in the presence of GLP. The kinetic characterization of mutants R100K, K340R, and E418A, which retain activity, is reported. Of those, R100K and K340R do not accumulate enzyme intermediate of enzyme-bound product under equilibrium conditions. These residues, while not essential for catalysis, are most likely important for substrate binding. All of the mutants are shown to be correctly folded by NMR spectroscopy.

5-Enolpyruvylshikimate-3-phosphate (EPSP)[1] synthase (EC 2.5.1.19) catalyzes the reaction between shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form EPSP, an intermediate in the biosynthetic pathway leading to chorismate, and hence to the aromatic amino acids (see Scheme 1). EPSP synthase is of interest as the primary target for the broad spectrum herbicide, glyphosate (*N*-(phosphonomethyl)glycine, GLP), which is the principal ingredient of Roundup. The mechanism of action and the structure of EPSP synthase have been studied extensively (*1*−*8*).

Several studies have identified residues of importance by chemical modification methods and mutagenesis (*9*−*17*). In this study, we attempt to address the role of the residues that had been identified previously as being close to the active site by chemical modification methods but have not been confirmed by mutation. The crystal structure of EPSP synthase in the absence of substrates is available (*5*). To date, no structure in the presence of substrates has been reported. NMR experiments in this laboratory are underway to obtain structural data in the presence of substrates and inhibitor. The identification of active-site residues that might aid in the structural analysis led to the mutagenesis program reported here.

A number of cationic residues have been proposed to be involved in substrate binding. K22 was identified by Huynh (*9*) using chemical modification with pyridoxal 5′-phosphate. Modification with *o*-phthalaldehyde implicated both K22 and K340 (*10*). R28 and R131 of the *Petunia hybrida* enzyme (R27 and R124 in *Escherichia coli*) were implicated by chemical modification and substrate protection studies using *p*-hydroxyphenylglyoxal (*13*). Selvapandiyan and co-workers (*16*) mutated R104 to R104K in the *Bacillus subtilis* enzyme (R100 in *E. coli*) and reported hypersensitivity to inhibition by glyphosate. K411 was also investigated because examination of the crystal structure suggests that the side chain sticks out into the postulated substrate-binding cleft region of the enzyme.

Three acidic residues are included in this study, as it has been proposed that acidic amino acids may be involved with binding of glyphosate, and E418 was identified using 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide (*11*). D384 is a candidate for mutagenesis based upon the conserved nature of this residue and position adjacent to H385, which has been implicated in substrate binding (*14*, *15*, *18*). D242 is another highly conserved acidic residue and was therefore also included as a candidate for mutagenesis.

MATERIALS AND METHODS

Expression and Purification of EPSP Synthase. E. coli EPSP synthase was overexpressed from the T7 promoter in a pET24b derivative, pWS230, using *E. coli* AB2829(λDE3) an *aroA*$^-$ strain. The mutant enzymes were purified by methods previously used for the wild-type enzyme (*19*). All EPSP synthases were judged to be at least 98% pure by SDS−PAGE.

Chemicals and Enzymes. All chemicals were of commercial reagent grade. S3P was purified from cultures of *Klebsiella pnemoniae* (*20*) by previously published methods (*19*). EPSP was synthesized enzymatically using wild-type EPSP synthase.

Mutagenesis. Mutagenesis was carried out using the Mutagene M13 in vitro phagemid mutagenesis kit (BioRad, CA) based on the method of Kunkel (*21*). *AroA* was cloned into pUC119 for mutagenesis. Oligonucleotides 17−21 bases

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[1] Abbreviations: DTT, dithiothreitol; EPSP, 5-enolpyruvylshikimate-3-phosphate; EPSPS, EPSP synthase; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; REDOR, rotational-echo double resonance; S3P, shikimate-3-phosphate.

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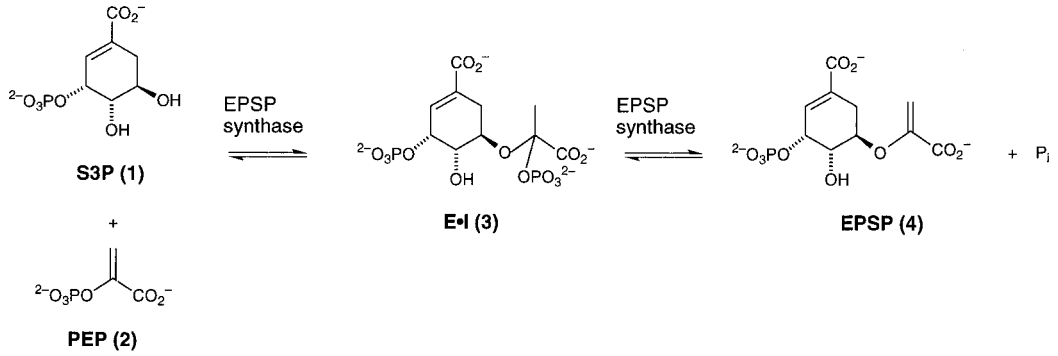
Scheme 1



S3P (1) + PEP (2) E•I (3) EPSP (4)

in length were used to change the desired codons. Mutations were confirmed by automated sequencing on an ABI 373 DNA sequencer (Applied Biosystems, CA) using dye terminator sequencing chemistry. Mutated *aroA* was cloned into pET24b with the ATG start codon into the *Nde*I site to express native protein.

^{15}N Labeling of EPSP Synthases. Overnight cultures of AB2829(λDE3) pWS250 with wild-type or mutant *aroA* were grown in M9 glucose medium containing $^{15}NH_4Cl$ and kanamycin (50 μg mL^{-1}) and supplemented with ^{15}N Celtone (10% v/v). After dilution 1:100 into fresh $^{15}NH_4Cl$ M9 the cells were grown to an OD_{600} of ∼0.5 and induced with 0.4 mM IPTG for 4 h. Purification of the labeled enzyme was identical to that of the unlabeled enzyme. The yield of purified, labeled enzyme was approximately 30 mg L^{-1} of media.

Determination of Kinetic Parameters. EPSP synthase was assayed routinely by the reverse coupled assay of Lewendon (*22*). Protein was determined by the Bradford (*23*) method with bovine serum albumin as a protein standard.

For kinetic studies, EPSP synthase was assayed in the reverse direction as noted above except that the assay buffer was 50 mM Tris−HCl, pH 7.0, 3 mM $MgCl_2$. In the forward direction, P_i release was measured using the EnzChek assay system (Molecular Probes, Eugene, OR). Cosubstrate concentrations were as follows: 250 μM S3P, 500 μM PEP, 100 mM P_i, and 50 μM EPSP. Glyphosate was used at 0.5 μM for apparent K_i determinations, which were calculated from V_{max} and K_M, assuming competitive inhibition. All assays were conducted at 25 °C. One unit of enzyme activity is the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product in 1 min. Kinetic data were analyzed using the program *Enzfitter* (*24*).

NMR Spectroscopy. High field Fourier transform (FT) NMR studies of EPSP synthase were performed on a Varian Inova 500 (11.75 T, 500 MHz ^1H) NMR spectrometer. Deuterium was used for locking the field. ^1H chemical shifts were referenced to external TSP at $\delta_H = 0.0$ ppm at 22 °C, and ^{15}N chemical shifts were calculated relative to this. ^{31}P chemical shifts were referenced to external 85% H_3PO_4 at $\delta_P = 0.0$ ppm at 22 °C. ^{13}C chemical shifts were referenced to external dioxane in D_2O buffer (δ_c= 67.4 ppm.). The HSQC spectra were obtained with the sensitivity-enhanced version of the HSQC pulse sequence (*25*) with a water flip-back pulse. Data were acquired with 16 scans over 8 kHz in D1 and 3.5 kHz in D2, with ^{15}N decoupling in D1. The pulse widths were 10.3 μs for proton and 29.5 μs for nitrogen. The data were processed with FELIX97 on a Silicon Graphics O2 workstation, with zero filling to 1024 points in D1 and 512 points in D2, solvent suppression, and a 90° phase-shifted sine-bell squared window function. Linear prediction was applied in D2.

Table 1: Specific Activity of Mutant Enzymes as a Percent of Wild-Type Activity

mutant	% wild type
WT	100
E418A	26
R100K	1.3
K340R	0.08
K22R	ND
K411R	ND
D384A	ND
R27A	ND
R100A	ND
D242A	ND

[a] ND: not detected, that is, below 0.01% wild type.

RESULTS AND DISCUSSION

Site-directed mutagenesis is a powerful technique that has been used effectively in the identification of residues involved in enzyme catalysis and substrate binding. In this study, several amino acids previously implicated by chemical modification studies have been mutated. A few of these residues have been mutated before in EPSP synthase enzymes from sources other than *E. coli.* All of these amino acids are highly conserved among the EPSP synthases from bacteria and plants (*26*). The mutant enzymes have been examined for their ability to successfully catalyze the conversion of S3P and PEP to EPSP and P_i. Several of these mutants were completely inactive, although those that retained activity were analyzed and the kinetic data is reported.

All mutant *E. coli* EPSP synthases were overexpressed from the plasmid pWS230 (*19*) in the *aroA*$^-$ *E. coli* host AB2829 (λDE3), and since AB2829 has no functional *aroA* gene, contamination with wild-type enzyme is not a concern (*14*). Mutations were made with attention to *E. coli* codon usage in order to avoid the use of rare codons. Mutant enzymes were overexpressed from the T7 promoter at a level comparable to that achieved for wild-type enzyme. Purification was identical to that described for wild-type enzyme (*19*) and the mutant enzymes identified by detection of enzyme activity or by SDS−PAGE.

Table 1 shows the specific activity of the mutants as a percentage of wild-type *E. coli* EPSP synthase. Several of

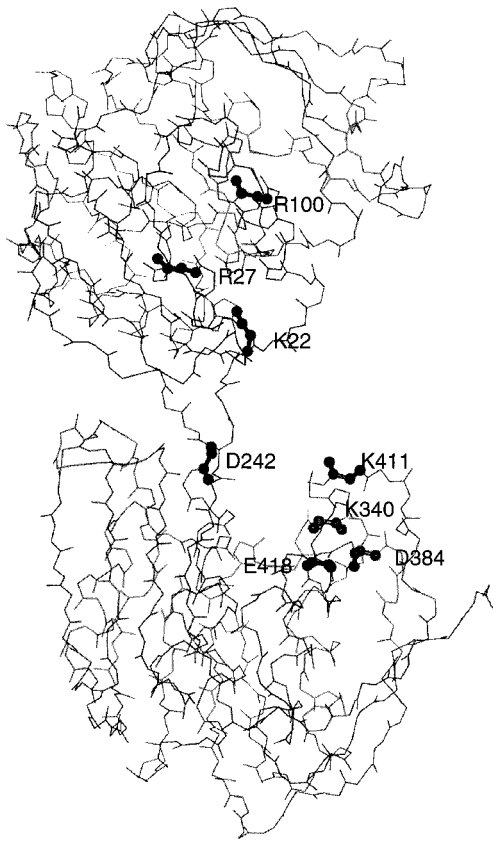


FIGURE 1: Structure of EPSP synthase (PDB file: 1EPS) with relevant active site residues highlighted.

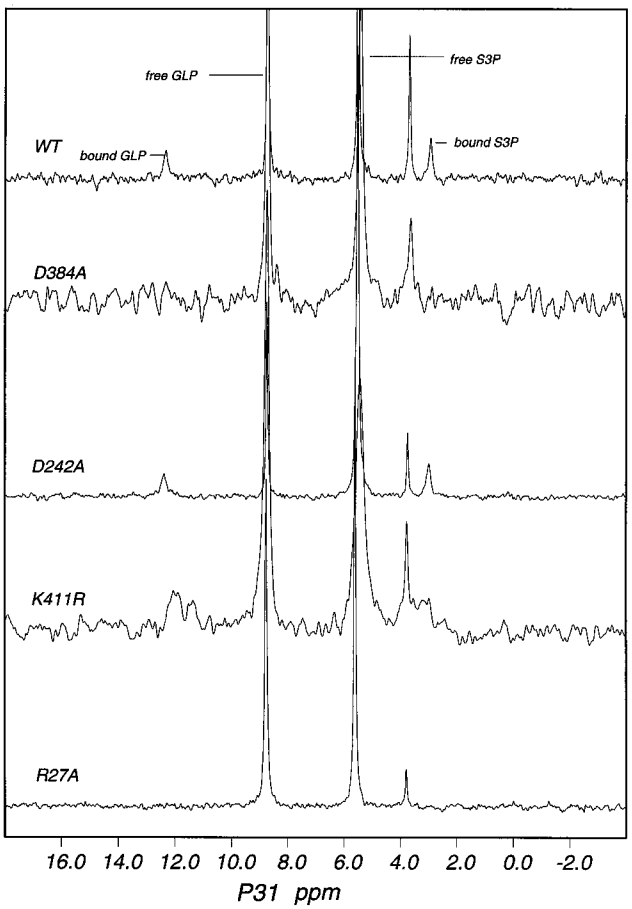


FIGURE 2: ^{1}H-decoupled ^{31}P solution-state NMR spectra at 22 °C of mutant and wild-type EPSP synthases (0.5−0.7 mM, 10 mM Tris−HCl (pH 7.8), 1 mM DTT, 0.02% NaN_3, and 8% D_2O), plus S3P (3.5 times [EPSPS]) and GLP (2 times [EPSPS]). The spectra were obtained with 6000 scans, a 5 μs pulse width, and a sweep width of 12 kHz. FIDs were Fourier transformed after linear prediction, zero filling to 8000 points with 10−20 Hz line broadening.

the mutant enzymes had no detectable activity indicating that the amino acid changes are not tolerated by the enzyme. In these cases it is possible that the mutated amino acids are essential for catalysis or substrate binding. However, there is always the possibility that the mutations may cause global conformational changes resulting in the lack of detectable enzyme activity.

K22R and K411R Mutants. Figure 1 shows the position of the residues in the crystal structure of the enzyme without substrates (*5*). The protein has an open structure, suggesting that it may close up upon substrate binding, as supported by the observation that a change in the tryptophan fluorescence only occurs upon binding the second substrate (or inhibitor), and by the observed changes in the "open" (*27*) and "closed" (*28*) structure of the related enzyme, uridine *N*-acetylglucosamine enolpyruvyl transferase. The residues mutated in the present study are located on the inside of the active site cleft that may form when the two domains close together. One of the goals of this laboratory is to measure distances within the enzyme−substrate/inhibitor complexes using a variety of structural methods including the solid-state NMR method rotational-echo double-resonance (REDOR) spectroscopy. This technique requires incorporation of stable isotopes at known positions in the protein that are within close proximity to the labeled substrates. Examination of the crystal structure reveals K22 and K411 to be pointing toward the proposed active site cleft and the region in which substrates bind. It was hoped that one or both of these lysine residues would tolerate a substitution to arginine. This would enable sequence-specific assignments to be made by comparison between uniformly ^{15}N-labeled wild-type and mutant enzymes, which would allow REDOR distance measurements to be made from the arginine ^{15}N to ^{31}P of S3P in the enzyme−S3P−GLP ternary complex. K22 has been implicated in substrate binding, and the equivalent residue of the *Petunia hybrida* enzyme has been mutated to K23R, K23A, and K23Q (*9*). Of those, K23R retained wild-type activity, whereas the other two mutations resulted in complete inactivation. Selvapandiyan and co-workers (*17*) have replaced K19 of the *B. subtilis* enzyme (equivalent to K22 in *E. coli*) with glutamic acid, and report that the mutant retained 0.5% of the wild-type specific activity. In the study of *E. coli* EPSP synthase reported here, no activity was detected for the K22R mutant, and thus it would appear that the *E. coli* enzyme is more sensitive to substitution at this position than either the *Petunia* or *Bacillus* enzymes. Interestingly, the K22R mutant does not bind either S3P or GLP, as indicated in Table 2, which reports ^{31}P chemical shifts for ^{31}P NMR spectra of a number of wild-type and mutant EPSP synthases in the presence of S3P and GLP (some of which are shown in Figure 2). The $^{1}H\{^{15}N\}$ HSQC spectra of the K22R mutant (data not shown) alone, and in the presence of S3P and GLP, were essentially indistinguishable from the HSQC spectra of the wild-type enzyme shown in Figure 3. HSQC spectra are extremely sensitive to changes in structure and can clearly indicate when a protein is

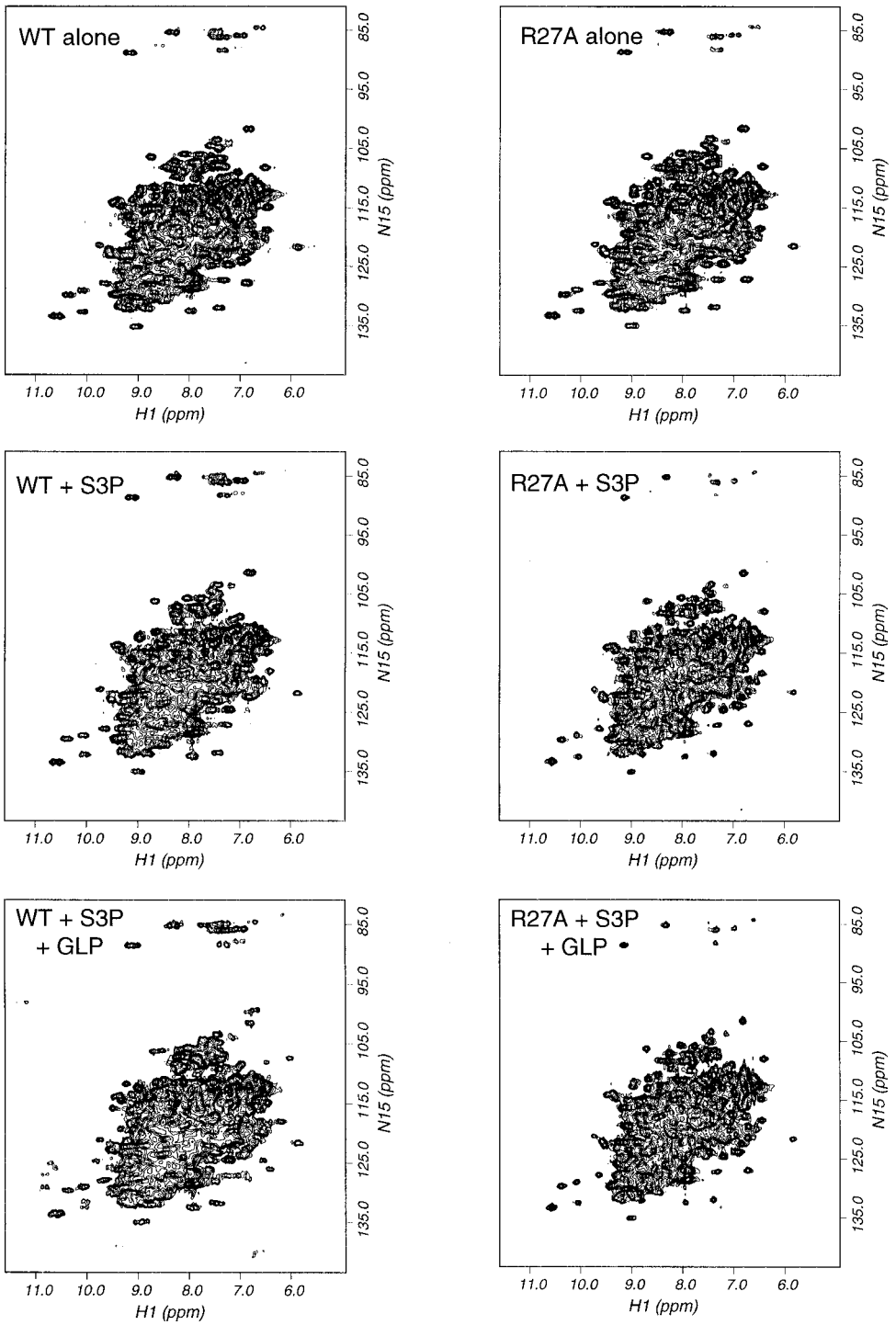


FIGURE 3: $^{1}H\{^{15}N\}$ 2D HSQC spectra at 22 °C of wild type and a representative mutant (R27A) EPSP synthases (0.5−0.7 mM, 10 mM Tris−HCl (pH 7.8), 1 mM DTT, 0.02% NaN_3, 8% D_2O), alone, with S3P (3.5 times [EPSPS], and with S3P and GLP (2 times [EPSPS]).

unfolded. For example, the HSQC spectrum of the wild-type enzyme in the presence of S3P results in only minor changes compared with the enzyme alone, whereas in the presence of both S3P and GLP, there are significant changes in resonance positions (some of which are under the large number of overlapping resonances in the middle of the spectrum, but can be detected in difference spectra). The HSQC spectra of the K22R mutant therefore show that the protein is folded correctly.

K411 is highly conserved and appears to be located at or near the active site of the enzyme (*29*). The K411R mutation

Table 2: ^{31}P Solution-State NMR Chemical Shifts (in ppm) of S3P and GLP with Wild-Type and Mutant EPSP Synthases

			GLP present		GLP absent	
protein	bound GLP	free GLP	free S3P	bound S3P	free S3P	bound S3P
WT	12.42	8.79	5.54	2.99	5.54	2.98
D384A		8.79	5.54		5.54	
D242A	12.43	8.8	5.58	3.04	5.5	
K411R	12.09	8.78	5.48	3.02	5.37	
R27A		8.82	5.66		5.66	
K22R	12.73	8.81	5.39	3.51	5.35	3.53

Table 3: Kinetic Parameters Determined for Active EPSP Synthase Mutants

mutant	K_M S3P (μM)	K_M PEP (μM)	K_i GLP (μM)	K_MPi (mM)	K_M EPSP (μM)
wild type	8 ± 4	13 ± 4	0.13 ± 0.05	5 ± 2	10 ± 5
R100K	21 ± 9	405 ± 150	0.24 ± 0.07	11 ± 8	84 ± 20
K340R	16 ± 2	56 ± 10	0.03 ± 0.02	56 ± 5	14 ± 5
E418A	7 ± 2	6 ± 2	0.03 ± 0.02	2 ± 0.2	10 ± 5

resulted in a complete loss of activity. This mutant appeared to bind S3P weakly, but only in the presence of GLP (see Figure 2 and Table 2). The $^1H\{^{15}N\}$ HSQC spectra of the K411R mutant (data not shown) alone, and in the presence of S3P and GLP were essentially indistinguishable from the HSQC spectra of the wild-type enzyme shown in Figure 3, indicating that the protein is folded correctly. On the basis of the position of residues K22 and K411 sticking into the active site cleft, it is possible that the positive charges are crucial for binding of the negatively charged substrates, with both playing an important role in S3P binding. If this is the case, the positioning of these positive charges and the number of hydrogen bonds involved must be critical, as substitution with arginine is not tolerated.

R27A, R100A, and R100K Mutants. R100 is highly conserved among known EPSP synthases and is in the putative PEP-binding site (*3*, *30*). Mutation of R104 to lysine in the *B. subtilis* (R100 in *E. coli*) enzyme introduced hypersensitivity to glyphosate (*16*). R27 is a highly conserved residue and is implicated by the modification of the equivalent R28 in the *P. hybrida* enzyme with phenylglyoxal and *p*-hydroxyphenylglyoxal. The addition of S3P and glyphosate completely protected the enzyme from modification (*13*). *Bacillus subtilis* R24 (*E. coli* R27) was mutated to aspartate resulting in a decrease in specific activity to 0.8% of wild type and a 10-fold increase in the K_M for S3P (*17*). Arginine residues involved in phosphate binding have been identified by our REDOR measurements (*6*), and changes to alanine will allow us to probe the significance of these residues. Changes to lysine allow us to probe the possibility of one versus two hydrogen bonds being involved in substrate binding, and make sequence-specific resonance assignments in the REDOR experiments with relatively conservative mutations. The R27A mutant was essentially inactive (Table 1), and did not bind either S3P or GLP (Table 2, and Figure 2). As shown in Figure 3, the $^1H\{^{15}N\}$ HSQC spectra of the R27A mutant alone, and in the presence of S3P and GLP, were essentially indistinguishable from the HSQC spectra of the wild-type enzyme, indicating that the protein is folded correctly. R100K retains 1% wild-type specific activity whereas the mutation to alanine had no detectable activity. The kinetic analysis (Table 3) of R100K revealed that the K_M for PEP increased 30-fold. The K_M values obtained for S3P and EPSP are also increased above that for wild type, though to a lesser extent. The K_i for glyphosate was found to be similar to that for wild type, and R100K does not show increased sensitivity to inhibition unlike the R104K *B. subtilis* mutant enzyme. The lack of correlation between glyphosate inhibition and PEP binding seen in *E. coli* R100K in this study has also been noted for other EPSP synthase mutants. G101A and P106S of the *Petunia* enzyme show little correlation between the K_i of glyphosate and K_M/k_{cat} of PEP (*3*). It has been recognized for some time (*4*, *31*) that the

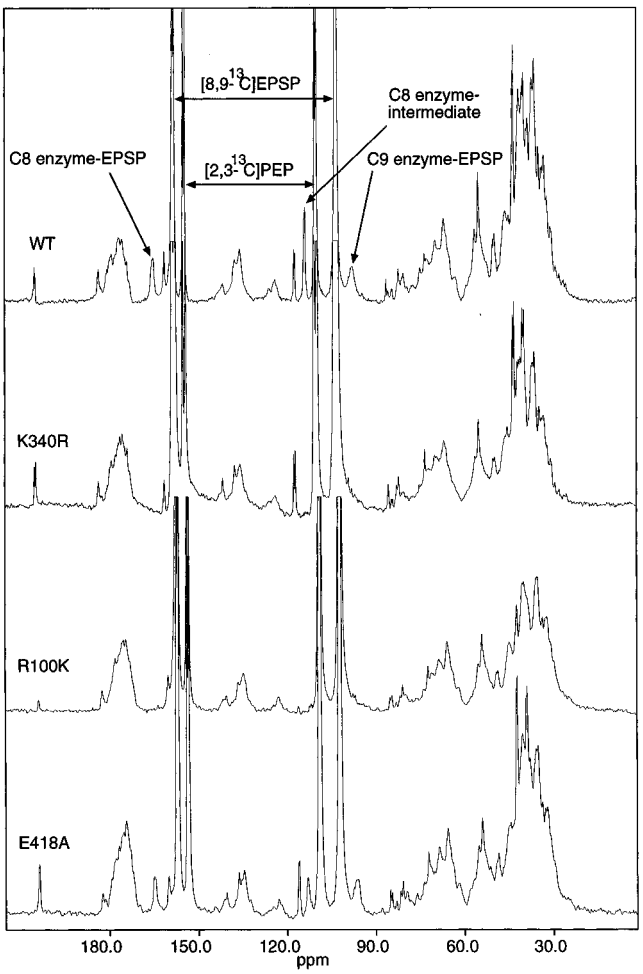


FIGURE 4: ^1H-decoupled ^{13}C solution-state NMR spectra at 4 °C of mutant and wild-type EPSP synthases (2 mM), 20 mM potassium phosphate (pH 7.0), 10 mM DTT, 10% D_2O, plus S3P (10 mM) and [2,3^{13}C] PEP (10 mM). The spectra were obtained with 30 000 scans, a 4 μs pulse width, and a sweep width of 35 kHz. FIDs were Fourier transformed after zero filling to 100 000 points with 50 Hz line broadening.

original proposal (*32*) that glyphosate acts as a transition-state inhibitor analogous to the PEP-derived carbocation formed in the enzymatic mechanism is unlikely. More recently it has been suggested that glyphosate does not compete with PEP for the same binding site on the enzyme but is competitive with respect to PEP for binding to the enzyme−S3P complex (*31*, *33*). The data reported here for R100K would support this hypothesis. A pH activity profile was obtained for R100K, and the mutant enzyme was found to be considerably more active at pH 6.0 than at pH 7.0 (150%, data not shown). This is different from the broad pH optimum at pH 7−8 seen with wild-type EPSP synthase (*14*). The data obtained in this study support previous studies, which suggest that R100 is located in the PEP-binding site. Figure 4 shows the solution-state ^{13}C NMR spectrum of the R100K in the presence of S3P and [1,2-^{13}C]PEP. The spectrum shows that resonances due to enzyme-bound intermediate and enzyme-bound EPSP are not present. Lack of accumulation of these species may be attributed to destabilization of both of these enzyme-bound species in the mutant enzyme. The conservative change retaining the positive charge at position 100 may decrease the binding of PEP but still allow some interaction with

substrate as reflected by the change in affinity for PEP. Abolishing the positive charge at this position, as is the case with the R100A mutant, may result in an inactive enzyme due to lack of substrate binding at this site.

K340R Mutant. K340 was implicated in the same chemical modification study that identified K22 (*34*). This residue lies in the fully conserved sequence: VKE. The activity of the K340R mutant is low compared with that of wild type (less than 0.1%). Kinetic analysis of K340R showed no change from wild-type K_M for S3P, K_M for EPSP, and the K_i for glyphosate. There was a 5-fold increase in K_M for PEP and a 10-fold increase in K_M for P_i. Unlike the mutant R100K, K340R showed a broad pH optimum at pH 7−8 as seen with wild-type enzyme (data not shown). The conservative mutation retaining the positive charge causes a considerable loss in activity that may be partly due to a decrease in ability to bind substrate, as well as being due to a loss in catalytic capacity as reflected in k_{cat}. Figure 4 shows the solution-state ^{13}C NMR spectrum of the K340R in the presence of S3P and [1,2-^{13}C]PEP. The spectrum shows that resonances due to enzyme-bound intermediate and enzyme-bound EPSP are not present. Again, lack of accumulation of these species may be attributed to destabilization of both of these enzyme-bound species in the mutant enzyme.

E418A Mutant. The proposed mechanism requires protonation of PEP at C3. H385 was initially proposed to be the acidic residue responsible, but mutational analysis of H385 showed it to be nonessential for activity (*14, 15*). It is possible that two acid/base residues are involved in PEP protonation and enzyme−intermediate deprotonation, as the addition−elimination reaction has been shown to occur with either anti-syn or syn-anti stereochemistry (not anti-anti or syn-syn) (*35*). This implies that the same residue cannot both deliver and remove the proton at C3 of PEP. The role of E418 has been suggested to be at or close to the glyphosate-binding site, either involved in the catalysis or interacting with and affecting the basicity of H385 (*11*). E418 is not conserved across the known EPSP synthase sequences but the acidic nature of the residue is conserved in all but the *Arabidopsis* enzyme where a glutamine residue is present. The negative charge on the glutamate or aspartate may interact with the positively charged amino group of glyphosate. Mutation of this residue to remove the charged amino acid at this site may identify the role of E418. E418A retains 25% of the activity of wild-type enzyme and has similar kinetic parameters. This suggests that this residue does not play an important role in catalysis or substrate binding. Glyphosate binding is not significantly altered, as reflected by the K_i values, and hence E418 cannot be critically located in the GLP-binding site. The loss of the charged group may be causing a local conformational change in the active site resulting in the decreased activity of the enzyme. Figure 4 shows the direct observation of the enzyme−intermediate complex by solution-state ^{13}C NMR spectroscopy. The spectrum of E418A is identical to that of wild type and shows the resonances assigned to enzyme-bound intermediate and enzyme-bound EPSP.

D384A Mutant. D384 is located in a fully conserved three amino acid sequence of EPSP synthase, DHR, which includes H385 that had been implicated in substrate binding previously and been the subject of mutagenesis studies (*14, 15, 18, 36*). D384 has not been implicated by any chemical modification studies but is of interest due to its proximity to H385, the conserved nature of the residue, and its potential role as an acid/base in catalysis. Removing the negative charge on D384 by mutation to alanine renders the enzyme completely inactive. This mutant does not bind S3P or GLP (see Figure 2 and Table 2). The $^1H\{^{15}N\}$ HSQC spectra of the D384A mutant (data not shown) alone, and in the presence of S3P and GLP, were essentially indistinguishable from the HSQC spectra of the wild-type enzyme shown in Figure 3, indicating that the protein is folded correctly. Mutations of H385 to alanine, glutamine, and asparagine did not completely inactivate the enzyme although substrate binding was reduced. This would imply that D384 is crucial for enzyme activity, and perhaps the changes in substrate affinity seen with the H385 mutants are due to steric effects on the adjacent residue. R386 has not been the subject of mutagenesis in this study. R386 is fully conserved among known sequences but was not implicated in the studies that specifically modified arginine residues.

D242A Mutant. D242 is fully conserved throughout the known EPSP synthase sequences and is therefore also a potential base in the enzymatic mechanism. The D242A mutant retains no detectable activity. This mutant appeared to bind S3P only in the presence of GLP (see Figure 2 and Table 2). The $^1H\{^{15}N\}$ HSQC spectra of the D242A mutant (data not shown) alone, and in the presence of S3P and GLP, were essentially indistinguishable from the HSQC spectra of the wild-type enzyme shown in Figure 3, indicating that the protein is folded correctly. The negative charge on this residue suggests that it may fulfill a similar role to that suggested for D384. However, disentangling the potential role of residues D384 and D242 as bases (or, conceivably, acids if their microscopic pK_a's are significantly perturbed), from their potential role as hydrogen-bonding partners for lysine and arginine residues involved in substrate phosphate or carboxylate binding in the "closed" conformation of the enzyme, is difficult to assess in the absence of detailed kinetic data (which cannot be obtained for an inactive enzyme).

SUMMARY

In summary, many of the mutations studied here resulted in complete loss of catalytic activity, thus suggesting that the residues are critically located in the active site of EPSP synthase. These residues may be essential for catalysis, though the possibility of local conformational changes cannot be ruled out without more detailed structural data. The HSQC data will reveal gross structural changes, such as global protein unfolding, and would reveal local structural changes only when the resolution of the NMR data is greatly improved through multiple labeling strategies. E418 can be ruled out as an essential residue as this mutant retains mostly wild-type characteristics. Most likely, the decrease in specific activity noted for E418A is due to local conformational changes around the active site rather than direct involvement of E418. R100 would appear to be important for PEP binding, as even a conservative change to lysine resulted in a significant decrease in affinity for the substrate. K340R retained little activity and showed decreased affinity for both PEP and P_i, so although not essential for activity, this conservative change was poorly tolerated by the enzyme. Finally, D384 and D242 both appear to have important roles in catalysis, though delineation of their precise role will have

to await structural analysis of the "closed" conformation of EPSP synthase.

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