Site-Directed Mutagenesis and NMR Studies of Histidine-385 Mutants of 5-Enolpyruvylshikimate-3-phosphate Synthase†

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ABSTRACT: The site-directed mutagenesis of His-385 of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase is reported. The steady-state kinetics for two mutants, H385Q and H385A, are compared with that of the wild-type enzyme. H385Q EPSP synthase was found to have 25% wild-type enzyme activity, whereas H385A EPSP synthase retained 1% activity. The K_M values for P_i and shikimate 3-phosphate were unaffected, whereas the K_M for phosphoenolpyruvate (PEP) was increased 10 times for H385Q EPSP synthase. The K_M for EPSP was unaffected in H385Q but raised by a factor of 10 in H385A EPSP synthase. The binding of glyphosate was studied by fluorescence spectroscopy and by ^{31}P NMR spectroscopy. Direct observation of the enzyme–intermediate complexes by ^{13}C NMR spectroscopy with [2,3-^{13}C]phosphoenolpyruvate was studied for the mutant enzymes and compared with the wild type. Under equilibrium conditions, H385A EPSP synthase does not accumulate enzyme-bound EPSP. These results suggest that, while critically located in the PEP binding site, His-385 is not the residue responsible for initiating catalysis through the protonation of PEP.

5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) catalyzes the reaction between shikimate 3-phosphate (S3P, **1**) and phosphoenolpyruvate (PEP, **2**) to form 5-enolpyruvylshikimate 3-phosphate (EPSP, **4**) 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, postemergence herbicide, glyphosate (*N*-(phosphonomethyl)glycine). The mechanism of action and the structure of EPSP synthase have been studied extensively (Barlow et al., 1989; Anderson & Johnson, 1990; Padgette et al., 1991; Evans, 1992).

Experiments carried out in this laboratory (R. J. Appleyard, P. N. Barlow, B. J. O. Wilson, and J. N. S. Evans, unpublished results) to determine the ionization states of S3P and glyphosate (GLP) when bound to EPSP synthase examined the pH dependence of the resonances of [3-^{13}C,^{15}N]glyphosate by ^{31}P, ^{15}N, and ^{13}C NMR spectroscopy. In the presence of EPSP synthase and GLP, the bound S3P phosphate no longer titrates over the pH range 6–10.5, and the resonance shifts to −2.0 ppm. The lack of titration suggests the proximity of a protonated amino acid side chain in the S3P binding site of the enzyme. One candidate is Lys-22, on the basis of chemical modification and mutagenesis studies (Huynh, 1988). Another is Arg-27, on the basis of chemical modification studies (Padgette et al., 1988). The bound glyphosate phosphonate also no longer titrates over the same pH range and the resonance shifts to 7.7 ppm, consistent with a dianionic species with the amino group partially ionized. An alternative suggestion by Castellino et al. (1989) is that the C–P–O bond angle changes upon binding to the enzyme and that this is responsible for the change in chemical shift rather than the ionization state of the glyphosate phosphonate.

The binding of glyphosate in an extended conformation has also been suggested by Christensen and Schaefer (1993) using solid-state REDOR NMR methods. That the phosphonate group does not titrate is consistent with the presence of an amino acid functional group in the glyphosate binding site close to the amino group of the glyphosate. Chemical modification studies of the protein implicated a number of residues as being at or in close proximity to this binding site: Cys-408 (Padgette et al., 1988), Glu-418 (Huynh, 1988), and an essential histidine (Huynh, 1987). Cys-408 was found to react with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of S3P and glyphosate to give inactive enzyme. However, cyanolysis of the DTNB-treated enzyme leads to an active thiocyano enzyme, indicating that the smaller group does not block the active site (Padgette et al., 1988). This would suggest that Cys-408 is close to the active site, but not required for catalysis. The enzyme mechanism requires the protonation of PEP at C-3 via an acidic or the conjugate acid of a basic enzyme amino acid side chain. Huynh (1988) suggested that His-385 may fulfill this role and that Glu-418 either may be involved directly with catalysis or may interact to affect the basicity of His-385.

While this paper was under review, Huynh (1993) published photooxidation studies of EPSP synthase suggesting that His-385 is essential and may be located in the glyphosate binding site. His-385 is the only histidine conserved throughout the known EPSP synthase sequences, as published by Griffin and Griffin (1991) (Table 1), and is within a conserved three-residue region: aspartate, histidine, and arginine. The X-ray crystal structure (Stallings et al., 1991) of EPSP synthase has been elucidated, and although it is not available in the public domain, it is known from the structure that His-385 is in the vicinity of the active site.

A combination of site-directed mutagenesis and classical biochemistry is the ideal approach to verify the role of His-385 in the active site. In this study, His-385 is replaced by both glutamine and alanine residues, and the purified mutant enzymes are compared with the wild-type protein by a number of methods. Glutamine was chosen as a conservative change with similar space-filling properties as histidine (Creighton,

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Scheme 1

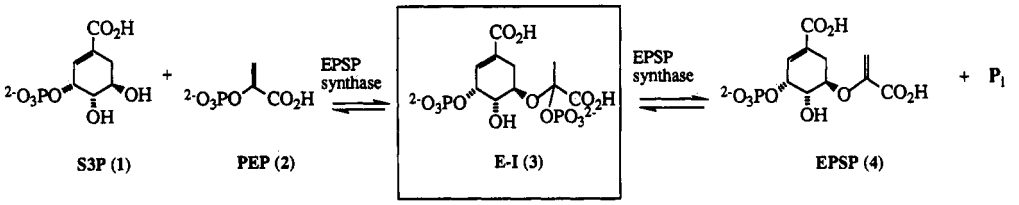

Table 1: Sequence Comparison of the Region around His-385 of *E. coli* EPSP Synthase

EPSP synthase source	sequence
Aspergillus nidulans	fcyd**DHR**vafs
Saccharomyces cerevisae	ctyd**DHR**vams
Arabidopsis thaliana	dtyd**DHR**mama
Lysopersicon esculentum	dtyd**DHR**mama
Petunia hybridia	dtyd**DHR**mama
Escherichia coli	atyn**DHR**mamc
Salmonella gallinarum	gtyn**DHR**mamc
Salmonella typhi	gtyn**DHR**mamc
Yersinia enterocolitica	gtyn**DHR**mamc
Bordetella pertussis	gtwd**DHR**mamc
Bacillus subtilis	sshg**DHR**igmm

1983; Schwartz & Dayhoff, 1979), while alanine, the most abundant amino acid, has no bulky side chains available for involvement in hydrogen-bonding or electrostatic interactions.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. AB2829 (*aroA*$^-$; Pittard & Wallace, 1966) and BL21 (Studier et al., 1990) are *Escherichia coli* K12 and *E. coli* B strains, respectively. The bacteriophage DE3 is a λ-derivative encoding T7 RNA polymerase. BL21(λDE3) is the usual pET vector bacterial host used for overexpression by the T7 RNA polymerase system. AB2829(λDE3) was constructed using a commercially available lysogenization kit (Novagen Inc., Madison, WI); the presence of λDE3 was confirmed by overexpression of *aroA*. Plasmid pWS230 is described elsewhere (Shuttleworth et al., 1992). Plasmid selection was maintained by the addition of antibiotics at the following concentrations: ampicillin (Ap), 100 μg mL^{-1}; kanamycin (Km), 50 μg mL^{-1}; and chloramphenicol (Cm), 30 μg mL^{-1}.

Chemicals and Enzymes. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) except where stated otherwise. S3P was purified from cultures of *Klebsiella pnemoniae* (Bondinell et al., 1971) by batch elution anion exchange chromatography on QAE Fast Flow (300 mM ammonium bicarbonate, pH 9.0). Further purification was achieved by FPLC on Mono-Q (Pharmacia/LKB, Piscataway, NJ) using a linear gradient of 10–500 mM ammonium bicarbonate (pH 9.0). Chorismate synthase was partially purified from *Neurospora crassa* using an adaptation of the procedure described by White et al. (1988). The partially purified enzyme was obtained by negative DEAE anion exchange chromatography followed by ammonium sulfate precipitation and FPLC on a Mono-Q anion exchanger. Protease inhibitors (1 mM PMSF and 1 mM benzamidine) were present throughout the purification. The resulting preparation had a specific activity of 2.9 and was judged to constitute ~10% total protein by SDS–PAGE. The enzyme was stored in 50% glycerol at −20 °C.

Mutagenesis. Mutagenesis was carried out using the Mutagene M13 *in vitro* mutagenesis kit (Bio-Rad, Richmond, CA) based on the method of Kunkel (1985). Using plasmid pWS230 as the starting material, 17-mer oligonucleotides were used to change the His-385 codon (CAC) of *aroA* to glutamine (CAG) and alanine (GCC) codons. The mutations were confirmed by DNA sequencing with the dideoxy method (Sanger, 1977) using [^{35}S]-α-dATP (1300 Ci mmol^{-1}, New England Nuclear, Boston, MA), Sequenase v.2.0 (U.S. Biochemicals, Cleveland, OH), and a primer (5′-TTGGC-TATTTATTGCCC-3′) complementary to the sequence 10 bases downstream of the *aroA* gene in pWS230.

Growth of Cells and Purification of EPSP Synthase. *E. coli* AB2829(λDE3) pWS230 was found to behave identically to *E. coli* BL21(λDE3) pWS230 in the overexpression of *aroA*. The cells were grown and the proteins were purified as described previously (Shuttleworth et al., 1992). All EPSP synthases were judged to be at least 98% pure by SDS–PAGE.

Determination of Kinetic Parameters. EPSP synthase was assayed routinely by the reverse coupled assay of Lewendon and Coggins (1983). Protein was determined by the Bradford (1976) method, with bovine serum albumin as a protein standard.

For kinetic studies, EPSP synthase (0.12 μM) was assayed in the reverse direction as noted above, except that the assay buffer was 50 mM Tris·HCl (pH 7.0), 50 mM KCl, and 2.5 mM $MgCl_2$. In the forward direction, EPSP synthase was assayed by coupling the reaction to that of chorismate synthase and continually measuring the formation of chorismate at 275 nm (Lewendon & Coggins, 1983). 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 (Leatherbarrow, 1987).

Fluorescence Studies. Fluorescence measurements were made on an SLM 4800 fluorometer; the excitation wavelength was 280 nm, and the emission wavelength was 360 nm. The decrease in fluorescence due to S3P and glyphosate binding was measured (Anderson et al., 1988). Glyphosate (to 50 μM) was titrated into a solution of 0.5 μM EPSP synthase plus 250 μM S3P in 50 mM HEPES (pH 7.0) and 50 mM KCl; after each addition of glyphosate, the fluorescence intensity was recorded as the average of 10 readings. Each titration was repeated three times. In all experiments, the data extended beyond that shown in the Results section. The data were fit to the following equation (Anderson et al., 1988)

using the program SigmaPlot:

$$F = F_0 + \frac{1}{2}\left(\frac{\partial F}{E_0}\right)\{(K_d + E_0 + S_0) - \sqrt{(K_d + E_0 + S_0)^2 - 4E_0S_0}\} \quad (1)$$

where F represents the observed fluorescence, S_0 is the glyphosate concentration, E_0 is the enzyme concentration, F_0 is the initial fluorescence, ∂F is the total change in fluorescence, and K_d is the dissociation constant.

pH–Activity Profile. The activities of the wild-type and H385Q EPSP synthases were determined as a function of pH using the reverse coupled assay in 100 mM P_i buffer (pH 5.4–8.4), 50 mM ammonium bicarbonate buffer plus 100 mM P_i (pH 8.0–9.0), and 50 mM succinate buffer plus 100 mM P_i (pH 5.0–5.6). The data were fit to the following equation (Turnbull et al., 1991):

$$\log y = C - \log\left(1 + \frac{[H]}{K_a} + \frac{K_b}{[H]}\right) \quad (2)$$

where y represents the activity, C represents the pH-independent value of the parameter, K_a and K_b are the pK_a values, and [H] is the hydrogen ion concentration.

Isotope Effects. Solvent isotope effects were determined by comparing the enzyme activity, by the reverse coupled assay, in protonated and deuterated phosphate buffer.

Substrate kinetic isotope effects were determined in the reverse direction using [^{2}H]EPSP. The deuterated EPSP was prepared enzymically from 10 mg of PEP and 5 mg of S3P with 40 units of EPSP synthase in deuterated 50 mM Tris·DCl (pD 7.6). After overnight incubation, the protein was precipitated with 70% ethanol, and the EPSP was purified from the supernatant by FPLC on Mono-Q (Pharmacia) using a linear gradient of 10–300 mM ammonium bicarbonate (pH 9.0). EPSP was determined by the perchloric acid phosphate assay (Chen et al., 1956). Mass spectroscopy determined the EPSP to be 68% doubly deuterated and 25% monodeuterated. The activities of the enzymes were determined by the reverse assay with protonated and deuterated EPSP.

NMR Spectroscopy. High-field Fourier transform (FT) NMR studies of EPSP synthase were performed on a Varian VXR-500S (11.75 T, 500 MHz, ^{1}H) NMR spectrometer. Deuterium was used for locking the field. Chemical shifts were referenced externally to samples of similar dielectric constant; ^{13}C NMR spectra were referenced to dioxane in D_2O buffer (δ_C = 67.4 ppm), and ^{31}P NMR spectra were referenced to H_3PO_4 in D_2O (δ_P = 0 ppm). Sample temperature was maintained at 4 °C using boil-off nitrogen. ^{13}C experiments were performed on EPSP synthase samples washed into 20 mM potassium phosphate, 0.4 mM DTT, and 10% D_2O (pH 7.0) with 2 mM EPSP synthase, 10 mM S3P, and [2,3-^{13}C]PEP. ^{31}P experiments were carried out in 50 mM Tris·HCl, 0.4 mM DTT, and 10% D_2O (pH 7.8) using EPSP synthase (2 mM), S3P (10 mM), and glyphosate (4 mM).

RESULTS

Mutagenesis. E. coli AB2829(λDE3) was chosen as the host strain, as it is deficient in the *aroA* gene and will contribute no EPSP synthase to the system; only the mutated plasmid encoded enzyme is present. AB2829(λDE3) behaves identically to the Studier (1990) host strain, BL21(λDE3); with respect to overexpression of the cloned protein, both strains accumulate EPSP synthase to ~50% total cell protein.

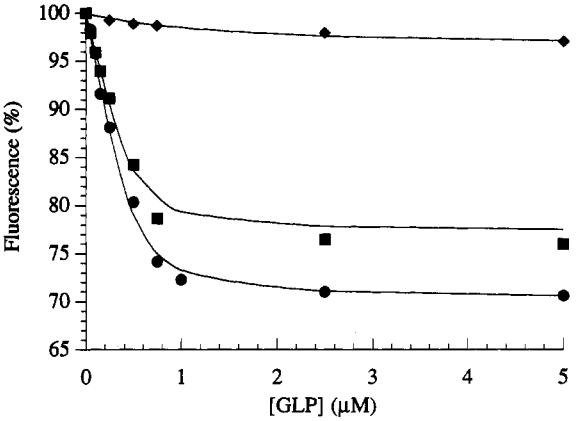

FIGURE 1: Fluorescence change associated with the EPSP synthase–S3P complex upon titration of glyphosate: ●, wild-type EPSP synthase; ■, H385Q EPSP synthase; ◆, H385A EPSP synthase. The data were fit to eq 1 described in the text.

Fluorescence Studies. Initial studies revealed H385Q EPSP synthase to have residual activity and H385A EPSP synthase to have no activity. It is known that upon binding of GLP to the EPSP synthase–S3P complex, there is a conformational change that results in a decrease in fluorescence at 360 nm due to a change in tryptophan fluorescence (Anderson et al., 1988). The change in fluorescence upon titration of GLP into a solution of EPSP synthase and S3P was determined for the two mutants and the wild-type enzyme. Figure 1 shows the results of these titrations. Most noticeable in this figure is the minimal change in fluorescence observed in the H385A mutant enzyme, where at 5 μM GLP the decrease in fluorescence was 3%, compared with 30% for the wild-type enzyme. Although not shown in Figure 1, the concentration of GLP was increased further to 55 μM with no additional decrease in the fluorescence for H385A EPSP synthase. H385Q EPSP synthase shows a change in fluorescence following the same pattern as the wild-type enzyme but to a lesser extent, leveling off at 75% of the starting intensity, compared with 70% for the wild-type enzyme. A fit of the data to eq 2 (described above) gave binding constants for GLP to the S3P–enzyme complex. The K_d for H385Q (0.055 ± 0.02 μM) was determined to be similar to that for the wild-type enzyme (0.063 ± 0.02 μM). The H385A EPSP synthase was determined to have a K_d of 1.1 μM (±0.5 μM).

^{31}P and ^{13}C NMR. ^{31}P solution-state NMR spectra of the EPSP synthases plus S3P and glyphosate show broad resonances representing enzyme-bound substrate and inhibitor. Figure 2 shows the 1D solution-state ^{31}P NMR spectra obtained for wild-type, H385Q, and H385A EPSP synthases. The resonances at 4.7 and 8.0 ppm are S3P and GLP, respectively. All three enzymes show resonances at 11.2 and 2.3 ppm, which are assigned to enzyme-bound GLP and S3P, respectively.

The ability of the two mutant EPSP synthases to form the intermediate was compared with that of the wild type, using the ^{13}C NMR spectroscopic method established in this laboratory for the direct observation of the enzyme–intermediate complex (Barlow et al., 1989; Evans, 1992). The enzymes were incubated with S3P and [2,3-^{13}C]PEP in the presence of a high concentration of phosphate (100 mM), such that the substrates and products were approximately equal and under steady-state equilibrium conditions, thus allowing the accumulation of any stable intermediates. The ^{13}C NMR spectra (Figure 3) show that, under these equilibrium conditions, both mutant EPSP synthases catalyze the reaction through to EPSP. The two resonances arising from

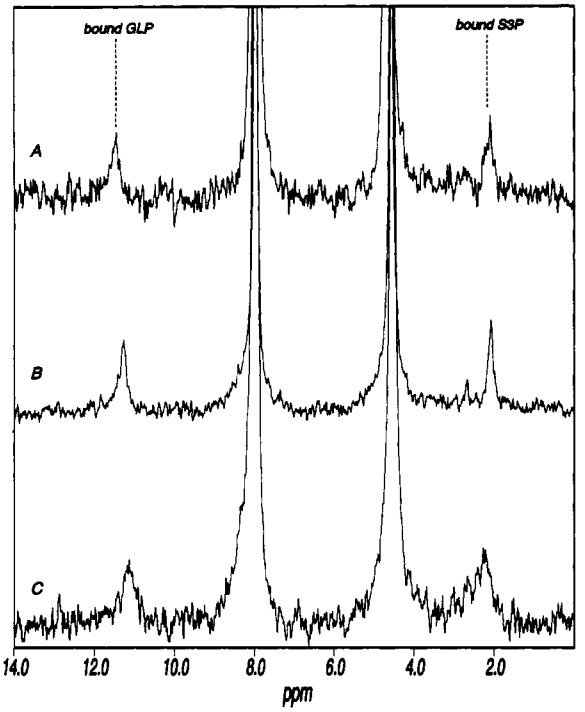

FIGURE 2: ^{31}P NMR spectra of wild-type, H385Q, and H385A EPSP synthases plus S3P and GLP: (A) wild-type EPSP synthase (2 mM), plus S3P (10 mM) and glyphosate (4 mM) in 50 mM Tris·HCl, 0.4 mM DTT, and 10% D_2O (pH 7.8); (B) H385Q EPSP synthase (conditions as in A); (C) H385A EPSP synthase (conditions as in A). All spectra were obtained with 2000 scans, a 0.2-s recycle time, an 8-μs pulse width, and 8K data points, and the FIDs were Fourier transformed with 5-Hz line broadening.

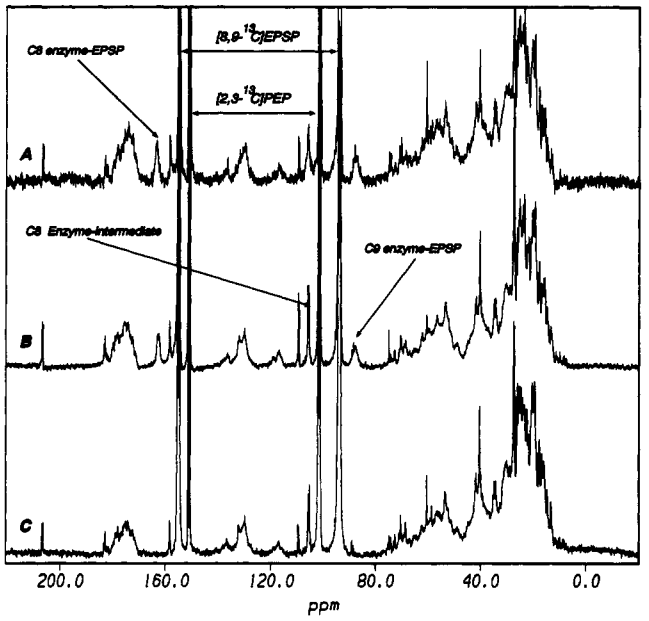

FIGURE 3: ^{13}C NMR spectra of wild-type, H385Q, and H385A EPSP synthases plus S3P and [2,3-^{13}C]PEP: (A) wild-type EPSP synthase (2 mM), 20 mM potassium phosphate (pH 7.0), 0.4 mM DTT, and 10% D_2O plus S3P (10 mM) and [2,3-^{13}C]PEP (10 mM); (B) H385Q EPSP synthase (conditions as in A); (C) H385A EPSP synthase (conditions as in A). All spectra were obtained with 36 000 scans, a 1.5-s recycle time, an 8-μs pulse width, and 32K data points, and the FIDs were Fourier transformed with 10-Hz line broadening.

EPSP are at 95.7 (C8) and 156.4 ppm (C9) [as assigned previously by Barlow et al. (1989)]. Activity assays of H385A EPSP synthase at high protein concentrations revealed that it was active, although at a level 100-fold lower than the wild-type (see Table 2). The ^{13}C NMR spectrum of H385Q EPSP synthase is very similar to that of the wild-type, but the H385A mutant spectrum lacks the resonances assigned to enzyme-bound EPSP at 88.9 (C9) and 164.8 ppm (C8), although it does show accumulations of enzyme-bound intermediate at 107.2 ppm (C8). The resonance due to C9 of the enzyme-bound intermediate at 24.5 ppm is masked by the natural abundance resonances of the protein.

Table 2: Comparison of Kinetic Parameters for Wild-Type, H385Q, and H385A EPSP Synthases

	wild type	H385Q	H385A
specific activity (units mg^{-1}) (reverse direction)	9.0	2.6	0.1
K_M P_i (50 μM EPSP)[b] (mM)	4.6 ± 1.4	10 ± 2.5	6.6 ± 1.5
K_M PEP (250 μM S3P) (μM)	34 ± 7	367 ± 17	ND[a]
K_M S3P (500 μM PEP) (μM)	27 ± 10	29 ± 13	ND[a]
K_M EPSP (50 mM P_i) (μM)	10 ± 5	4 ± 2	89 ± 10
K_i glyphosate (200 μM S3P) (μM)	0.1 ± 0.05	2.8 ± 0.5	ND[a]

Kinetic Isotope Data	wild-type V_H/V_D	H385Q V_H/V_D
[^2H]EPSP	1.53 ± 0.17	1.67 ± 0.13
D_2O solvent (reverse assay)	1.67 ± 0.16	1.33 ± 0.07

[a] ND, not determined. [b] Cosubstrate concentration is in parentheses.

Kinetic Data. The specific activity of H385A EPSP synthase was determined to be 0.1 units mg^{-1}, approximately 1% of the wild-type activity. Kinetic parameters obtained for the mutants are shown compared to those for the wild-type enzyme in Table 2. The K_M values for S3P and EPSP are unaltered for H385Q EPSP synthase, whereas the K_M for PEP is a factor of 10 higher than that for wild type. The K_i for GLP is increased almost 30-fold in H385Q EPSP synthase. K_M values for the forward direction could not be obtained for H385A EPSP synthase due to the low activity. In the reverse direction, K_M for P_i was similar to that for wild type, whereas the K_M for EPSP was a factor of 10 higher.

Isotope Effects. The solvent and substrate deuterium isotope effects on H385Q EPSP synthase were investigated with respect to wild type (Table 2). The results are shown as the ratio V_H/V_D to allow meaningful comparison. In the reverse direction, the substrate isotope ([^2H]EPSP) effects were identical to those of the wild-type EPSP synthase (within experimental error). There is a small difference between the solvent isotope effects measured for the mutant and those for the wild-type enzymes.

pH Profile. The pH activity profiles for the H385Q and wild-type EPSP synthases are shown from pH 5.4 to 8.5 in Figure 4. Both enzymes show two pK_a's, as depicted by the bell-shaped curves. Due to the scatter of the data, it is difficult to determine whether the wild-type enzyme is showing two pH optima. The striking point to note is that the pH profile has shifted dramatically for the H385Q EPSP synthase, from an optimum at pH 7.6 in the wild type to pH 6.5 in the mutant. A fit of the data to eq 2 (see above) results in pK_a values of 6.4 and 9.0 for the wild-type enzyme and 5.6 and 7.5 for the H385Q EPSP synthase. Additional data extending the pH range (not shown) suggest a second pH optimum at a lower pH (around pH 5.5) for the wild type. The EPSP synthase from *Klebsiella pneumoniae* also shows two pH optima (Steinrucken & Amrhein, 1984).

DISCUSSION

His-385 was implicated as being close to the PEP binding site by Huynh (1987), and it was suggested that this histidine residue may be responsible for the protonation of PEP at C-3. The purpose of this study was to investigate this suggestion

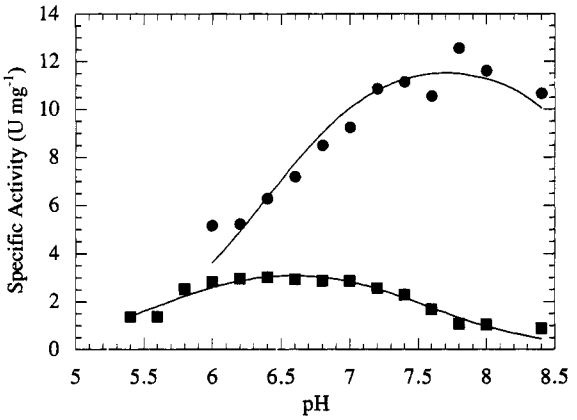

FIGURE 4: pH–activity profile for wild-type and H385Q EPSP synthases: ●, wild-type EPSP synthase specific activity measured by the reverse assay; ■, H385Q EPSP synthase (measured as above). The data were fit to eq 2 described in the text.

by mutating His-385 and characterizing the mutant proteins. Two mutant EPSP synthases were investigated, H385A and H385Q. Glutamine is a conservative substitution for histidine that can resemble histidine as a hydrogen bond donor, thus minimizing perturbations in the three-dimensional structure. Alanine has no reactive side chains to contribute to catalysis or hydrogen bonding.

In a study of this nature, it is important to determine that the mutations required are the only changes in the protein. To address this issue, the mutated genes were sequenced throughout and found to contain only the intended base changes. Alteration of His-385 to glutamine and alanine did not result in the complete loss of enzyme activity, although H385A EPSP synthase retained only 1% of the original activity. It is also necessary to determine that the mutant enzymes are pure and that the activity determined is not due to the unlikely possibility of a small amount of contaminating wild-type enzyme that may have been acquired during the enzyme preparation. The use of the *aroA*$^-$ host strain rules out any wild-type enzyme in the *E. coli* cells, and furthermore, comparison of the kinetic parameters of the mutant EPSP synthases with wild type reveals that the K_M values have altered. Differing K_M values are proof that the mutant enzyme is not contaminated with exogenous wild-type enzyme, as this parameter is not affected by the amount of enzyme present; it measures only active enzyme.

Recently, extensive kinetic studies of EPSP synthase were published by Gruys et al. (1992, 1993), and kinetic parameters for this enzyme have also been determined by Duncan et al. (1984) and Huynh (1987). The parameters determined here are in agreement with these authors, with the exception of the K_M value for S3P, which was determined here to be 27 μM. This value is similar to that determined by Huynh (1987), who reported 20 μM. In contrast, Duncan et al. (1984) and Gruys (1992) reported lower values: 2.5 and 3.6 μM, respectively. The difference in this determined parameter may arise from the quantitation of S3P, which in our experience has been subject to variation. In all of the experiments reported here, the mutant enzymes are compared with the wild-type enzyme under similar conditions to minimize any variations in the substrate concentrations. The K_M values obtained for S3P show the H385Q enzyme to be similar to the wild type, and the K_M values for P_i are similar to the wild type for both mutants. However, the K_M for PEP for the H385Q enzyme is 10 times that for the wild type. Glyphosate is a competitive inhibitor with respect to PEP, and hence it would be reasonable to expect that a mutation resulting in an increased K_M for PEP should cause an increase in the K_i for glyphosate. The K_i for glyphosate was found to be increased by almost 30-fold (from 0.1 to 2.8 μM) for the H385Q EPSP synthase compared with the wild-type enzyme. The H385A enzyme has a K_M for EPSP almost 10 times higher than those for the wild-type or H385Q enzymes. This suggests that in the H385A enzyme the binding site or local conformation is disrupted sufficiently to affect the binding of EPSP, whereas this is not the case for H385Q. It would appear that the binding sites for S3P and P_i are not affected by the mutations at His-385, whereas those for PEP and EPSP are altered. Clearly, His-385 cannot be the sole residue responsible for the protonation of PEP at C-3 as both mutants retain enzyme activity, with the more conservative H385Q mutant having 25% of the wild-type activity.

The binding of glyphosate to the enzyme–S3P complex is usually associated with a decrease in the tryptophan fluorescence, although this decrease was not observed for the H385A EPSP synthase. The conformational change believed to occur upon the binding of substrates must be hampered in the H385A mutant, as the enzyme retains 1% of the wild-type activity and the ^{31}P NMR data show enzyme-bound S3P *and* glyphosate. Interestingly, Huynh (1987) reported fluorescence changes upon the binding of S3P and glyphosate to diethyl pyrocarbonate (DEPC) modified EPSP synthase, implying that the chemical modification of the enzyme did not affect the tryptophan fluorescence. That the chemical modification did not result in a complete loss of activity was explained as being due to the instability of the DEPC, but the residual activity reported here for the H385A EPSP synthase suggests that this was not the case. The H385Q EPSP synthase does show a 25% decrease in fluorescence intensity upon binding glyphosate, compared with a 30% decrease for the wild-type enzyme. This supports the finding that the K_i for glyphosate is increased for the H385Q mutant. However, the K_d for glyphosate was found to be very similar for both the wild type and H385Q EPSP synthase, and this suggests that binding of the glyphosate is not altered, but that inhibition of enzyme activity is affected. The K_d values determined from the fluorescence must be treated with caution due to the possibility of a conformational perturbation in the mutant enzymes that reduces the tryptophan fluorescence and therefore the measured parameter for the K_d computation. The H385A mutation, which is less conservative, might be expected to have a more significant effect on the protein conformation. The lack of fluorescence change upon addition of the glyphosate to the H385A enzyme–S3P complex might be due to perturbations in the protein structure rather than loss of the ability to bind glyphosate. The K_d for GLP for the H385A EPSP synthase was calculated to be 1.1 μM, which is approximately 20 times greater than that for wild type, although given the argument for a possible conformational change, this value must be regarded with caution. The ^{31}P solution-state NMR spectra of the enzymes plus S3P and GLP (Figure 2) show enzyme-bound S3P and GLP for both mutants. Enzyme-bound substrate and inhibitor are seen at δ_P = 2.3 and 11.2 ppm, respectively. This is consistent with our solid-state REDOR NMR results, which suggest that H385A EPSP synthase is capable of binding glyphosate (Y. Li, W. A. Shuttleworth, L. McDowell, J. Schaefer, and J. N. S. Evans, unpublished results).

Direct observation of the enzyme–intermediate complex by solution-state ^{13}C NMR shows that both mutant enzymes are able to form the intermediate. The spectrum obtained for H385Q EPSP synthase is identical to that of the wild-type

enzyme, but the spectrum obtained for H385A EPSP synthase does not show the resonances assigned to enzyme-bound EPSP. Clearly, the kinetic data that show that H385A EPSP synthase has a greatly increased K_M for EPSP compared with H385Q and wild-type enzymes provide evidence that, under steady-state equilibrium conditions, the bound EPSP species would not be expected to accumulate at concentrations detectable by ^{13}C NMR spectroscopy. Therefore, it is reasonable to conclude that the enzyme-bound EPSP complex is destabilized in the H385A mutant relative to the wild-type enzyme. The enzyme-bound intermediate complex must still be relatively stable, as this is detectable in the spectrum at 107.2 ppm, and the fact that it accumulates for H385A EPSP synthase in the absence of bound EPSP is not remarkable considering that the experiment was conducted at equilibrium. In Figure 3 the line widths for the bound intermediate are sharper than those of the bound EPSP, as was observed previously (Barlow et al., 1989). This is due to the fact that line width is a function of the exchange rate of the enzyme-bound species.

H385Q EPSP synthase retains 25% of wild-type activity, and it is conceivable that the replacement of histidine by glutamine could create a "hole" in the active site, since the volume of the glutamine residue is less than that of the histidine. Histidine has a volume of 153.2 Å^3, while glutamine has a volume of 143.9 Å^3 (Creighton, 1983). Although this change is not sufficient to accommodate a water molecule, for example, which has a volume of 29.8 Å^3, additional local conformational adjustments conceivably could generate the additional 20.5 Å^3 required. Under such circumstances, the glutamine residue could recruit the water molecule to protonate the PEP in a manner somewhat similar to that proposed for the E165D mutant triosephosphate isomerase by Raines et al. (1986). To test this hypothesis, the solvent isotope effects for the wild-type and mutant enzymes were compared using deuterated and protonated solvents. Absolute values cannot be compared, but the V_H/V_D ratio for the enzymes can be considered. The enzymes do not show identical solvent deuterium isotope effects, but we would expect to see a much larger effect if a water molecule was being incorporated into the mechanism. Therefore, it is unlikely that H385Q EPSP synthase is recruiting a water molecule to protonate the substrate.

The pH profiles of the wild-type and H385Q EPSP synthases differ considerably. The wild-type enzyme has an optimum at pH 7.6, whereas the mutant enzyme shows a broad optimum centered around pH 6.4. Substrate ionizations can be ruled out on the basis of work conducted previously in this laboratory (R. J. Appleyard, P. N. Barlow, and J. N. S. Evans, unpublished results). The pK_a at 6.5 in the wild-type enzyme might be due to ionization of His-385, since histidine imidazolium pK_a values in proteins are normally in the range 5.6–7.0 (Fersht, 1985). Clearly, if this were the case, we would expect to see a change in the mutant proteins, which is certainly true for H385Q since this protein has pK_a values of 5.6 and 7.5. However, a pK_a of 6.5 is also consistent with an acidic residue, since pK_a values can shift substantially depending on the environment (Inoue et al., 1992). Thus, another explanation is that the shifts in pK_a values in the mutant reflect perturbations in the pK_a of this acidic residue, which result from changes in the electronic environment due to the removal of His-385. Therefore, interpretation of the pH profiles alone is insufficient to establish the role of His-385.

His-385 clearly is an important residue in EPSP synthase, as is shown by the H385A mutant enzyme which retains only 1% of the wild-type activity. The remaining 25% activity in the H385Q mutant enzyme suggests that this is not the base responsible for the protonation of PEP at C3. Glutamine may be considered to be stereochemically equivalent to the N$^\epsilon$H of histidine (Leatherbarrow & Fersht, 1987), and therefore H385Q is a conservative substitution for this group. Likewise, the amide NH_2 of asparagine is considered to be a conservative substitution for the N$^\delta$H of histidine. Neither of these amino acids could protonate the substrate, but the 25% remaining activity present in the H385Q EPSP synthase may be due in part to the stabilizing effect of H-bonding from the amide NH_2 of glutamine, mimicking the effect of the N$^\epsilon$H of histidine. It will be interesting to compare the mutant H385N EPSP synthase with the H385Q and wild-type enzymes. We suggest that His-385 is close to the active site of the enzyme, a suggestion also corroborated by the X-ray crystal structure of the unliganded enzyme, but that another residue is responsible for the protonation step. The kinetic studies suggest that His-385 is important for substrate binding, in agreement with the recently published photooxidation studies of Huynh (1993). In any of these studies, it is necessary to remain cautious about the possibility of gross conformational changes occurring until these can be ruled out by structure determination.

It is possible that there might be two residues involved, as the addition–elimination reaction has been shown (Grimshaw et al., 1982, 1984; Lee et al., 1984) to occur with either *anti-syn* or *syn-anti* stereochemistry (but not *anti-anti* or *syn-syn*), implying that the same residue cannot both deliver and remove the proton at C3 of PEP. Another possibility is that the phosphate anionic oxygen of the intermediate might deprotonate itself in an intramolecular reaction involving a 6-membered-ring transition state. Work is currently underway in this laboratory to identify other likely residues that may be involved in the catalytic mechanism.

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