^{31}P, ^{15}N, and ^{13}C NMR of Glyphosate: Comparison of pH Titrations to the Herbicidal Dead-End Complex with 5-Enolpyruvoylshikimate-3-phosphate Synthase†

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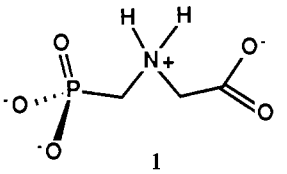
ABSTRACT: The herbicidal dead-end ternary complex (E^{S3P}_{Glyph}) of glyphosate [*N*-(phosphonomethyl)glycine] with 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) and the substrate shikimate 3-phosphate (S3P) has been characterized by ^{31}P, ^{15}N, and ^{13}C NMR. The NMR spectra of EPSPS-bound glyphosate show unique chemical shifts (δ) for each of the three nuclei. By ^{31}P NMR, glyphosate in the dead-end complex is a distinct species 3.5 ppm downfield from free glyphosate. This signal is modeled by free glyphosate alone in solutions of pH 10.1 and −0.8 as shown by the ^{31}P δ vs pH dependence. The ^{13}C signal [for the C-1 [^{13}C (99%)]] of glyphosate in the dead-end complex is shifted 4 ppm downfield from that of free glyphosate. This is not consistent with protonation of the carboxylate since the ^{13}C δ vs pH dependence dictates a model pH of 10.1. The ^{15}N signal for glyphosate (99%) in the dead-end complex is 5 ppm further downfield than that of any free zwitterionic species and 10 ppm downfield from that of the average free species at pH 10.1. The ^{15}N δ for glyphosate in the dead-end complex requires that the amine be fully protonated. Hence, the phosphonomethyl and carboxymethyl moieties of glyphosate must have orientations similar to that of glyphosate free in solution at pHs 10.1 or −0.8. The structures of each ionic state of glyphosate are modeled with force field calculations by using MacroModel. The modeled glyphosate −3 and +1 net charged species were found to be very similar and distinctly different from the 0, −1, and −2 species, which are predicted to form intramolecular H-bonds. A correlation is made for the ^{31}P δ and the C–P–O bond angle, and the ^{13}C and ^{15}N δ values are postulated to be related to C–C–O and C–N–C bond angles, respectively. The downfield ^{31}P chemical shift perturbation for S3P ($\Delta\delta$ = 0.54) in the EPSPS binary complex is consistent with ionization of the 3-phosphate of S3P upon binding. However, an upfield $\Delta\delta$ of 1.94 occurs when glyphosate binds to the binary complex, forming the dead-end complex. Comparison with the S3P ^{31}P δ vs pH titration curve specifies predominantly the dianion of the 3-phosphate in the E^{S3P} binary complex, while the E^{S3P}_{Glyph} complex indicates net protonation at the 3-phosphate. Chemical shift perturbations of this latter type may be explained by changes in the O–P–O bond angle [Gorenstein, D. G. (1984) *Phosphorus-31 NMR, Principles and Applications* (Gorenstein, D. G., Ed.) pp 1–33, Academic Press, Orlando, FL].

The sensitive chemical shift dependence of ionizable substituents facilitates the study of enzyme–ligand interactions by NMR. Glyphosate, containing three different ionizable NMR active nuclei, provides a unique opportunity in a small molecule to determine the interrelationship of each of the moieties. This study probes by NMR the nature of the ionization state of glyphosate's amine and the local environments of the phosphonate and carboxyl while bound to the enzyme in the dead-end ternary complex with S3P (E^{S3P}_{Glyph}). Glyphosate's binding site is suggested to be near or in the catalytic site of EPSPS,[1] but the exact nature of the interaction is not yet characterized in detail.

Glyphosate [*N*-(phosphonomethyl)glycine] (**1**) is the active ingredient in the formulation of the broad-spectrum herbicide Roundup, discovered by Franz (1985) and shown to target EPSP synthase (Amrhein et al., 1980; Steinrucken & Amrhein, 1980), the sixth step (Scheme I) in aromatic amino acid biosynthesis (Levin & Sprinson, 1964). Glyphosate's kinetic inhibition of the enzyme has been characterized as competitive with PEP by Steinrucken and Amrhein (1984) and uncompetitive with S3P according to Mousdale and Coggins (1984). The enzyme has since been shown by Anderson et al. (1988b) to require a compulsory binding order with S3P binding first and PEP second. Glyphosate binds with a K_d = 0.16 μM for



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[1] Abbreviations: AMPA (aminomethyl)phosphonate; A_{280nm}OD, optical density at 280 nm; $\Delta\delta$, change in chemical shift; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EPSP, 5-enolpyruvoylshikimate 3-phosphate; S3P, shikimate 3-phosphate; EPSPS, 5-enolpyruvoylshikimate-3-phosphate synthase; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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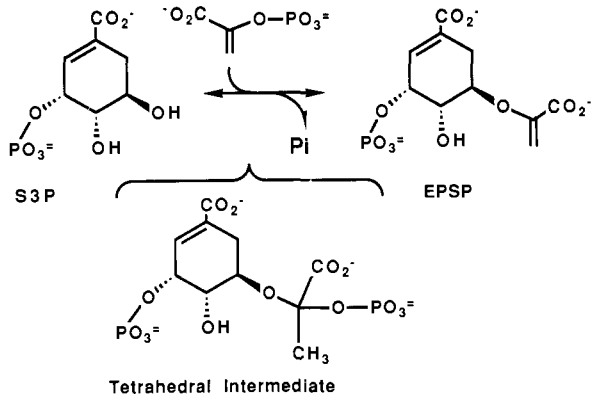
Scheme I

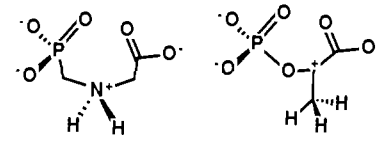


the *Escherichia coli* EPSPS and requires the preformed E^{S3P} as determined from measuring the change in the intrinsic fluorescence upon glyphosate binding. Anderson et al. (1988a) showed that no fluorescence change is seen for S3P binding, but EPSP association does induce a small fluorescence change. EPSP synthase in petunia is a preprotein containing a transit peptide that is directed to the chloroplast (della-Cioppa et al., 1986). Glyphosate inhibits the import rate of the pre-EPSPS across the chloroplast membrane by 80% (della-Cioppa et al., 1988). The stability of E^{S3P}_{Glyph} formed with glyphosate in the cytoplasm is postulated to interfere with the transport of pre-EPSPS. Since glyphosate inhibition is uncompetitive with S3P, then as S3P builds up in vivo, the equilibrium locks EPSPS into the dead-end complex. Cornish-Bowden (1986) has commented that uncompetitive inhibitors, if acquired in concentrations approaching their K_is, will always be fatal, hence the lack of naturally occurring uncompetitive toxins.

UDP-*N*-acetylglucosamine enolpyruvoyl transferase (EC 2.5.1.7), the only other enzyme known to catalyze the transfer of the carboxyvinyl group of PEP, is proposed to utilize a covalent carboxyvinyl intermediate (Zemell & Anwar, 1975). This enzyme is not inhibited by glyphosate, but is irreversibly inactivated by the antibiotic phosphonomycin (Kahan et al., 1974) through modification of an active-site cysteine. EPSPS, on the other hand, is not affected by phosphonomycin, but does contain two readily modified cysteines (Padgette et al., 1988a). Cysteine 408 is protected from DTNB when EPSPS is in the dead-end complex with glyphosate, but is not completely protected from *N*-ethylmaleimide. Other "active-site" amino acids are identified that can be protected from modification by the dead-end complex. Lysine 22 can be modified by pyridoxal phosphate, but is protected by S3P–glyphosate or just EPSP (Huynh et al., 1987). A histidine residue is also implicated at the active site of the *E. coli* EPSPS (Huynh, 1988). Arginines 28 and 131 in the petunia EPSPS are protected from phenyl glyoxal modification by the dead-end complex (Padgette & Kishore, 1988b). Of the three reactive arginines, one is protected by S3P alone, while approximately two are protected by EPSP alone. The protection afforded by EPSP alone and the intrinsic fluorescence found upon EPSP binding suggest that glyphosate overlaps with the carboxyvinyl group and that glyphosate could be a transition-state inhibitor of EPSPS. Mutant EPSPSs are reported to be glyphosate tolerant where the K_d of glyphosate is raised, but these enzymes also suffer an increased K_m for PEP (Stalker et al., 1985; Kishore et al., 1986). Collectively, these findings support glyphosate's binding site in the dead-end ternary complex to be near or in the catalytic site of the enzyme.

Mechanistically, the original work by Bondinell et al. (1971) demonstrated that C–O bond cleavage occurs in PEP and there is reversible protonation of the C-3 position of PEP. In the interim several investigators (Grimshaw et al., 1982, 1984; Anton et al., 1983; Lee et al., 1984; Asano et al., 1985) have confirmed these results by demonstrating that protonation occurs with substantial isotope effect, resulting in stereochemical transfer of the carboxyvinyl group. It was also shown that protonation of PEP may occur without hydrolysis in the presence of 4,5-dideoxyshikimate 3-phosphate (Anton et al., 1983). Bondinell et al. (1971) postulated that the reaction occurred by direct attack of S3P on PEP to form a 5-[2′-(phosphopyruvoyl ketal)]shikimate 3-phosphate as a tetrahedral intermediate during the transfer of the carboxyvinyl group to the 5-hydroxyl of S3P to form P_i and EPSP.

Chart I



This direct-addition-elimination mechanism led to the hypothesis that glyphosate acts as a transition-state inhibitor where the ammonium cation mimics the C-3 protonated PEP carbocation with each of the charged groups occupying like positions (Chart I). Binding and kinetic studies by Anderson et al. (1988a) suggest that glyphosate is not a particularly good transition-state inhibitor. The most recent pre-steady-state kinetic studies (Anderson et al., 1988b) propose a tetrahedral intermediate as postulated by Bondinell et al. (1971), but leave the question open as to the exact nature of glyphosate inhibition.

The mechanism of glyphosate inhibition of EPSPS is not fully described for the molecular level mode of action. This NMR study was directed at elucidating more characteristics of this herbicidal dead-end complex and particularly how and why glyphosate interacts so specifically with the enzyme. To aid the interpretation of the chemical shift perturbations induced by the enzyme, the ^{13}C, ^{15}N, and ^{31}P chemical shifts of glyphosate are cataloged through the entire pH range. The opposite effects of pH on the sign of the $\Delta\delta$ for phosphonates vs phosphates are discussed. Structures for each of the ionization states of glyphosate are predicted and minimized by using MacroModel. These structures are then related to the interpretation of the $\Delta\delta$ observed for each nucleus in the respective pH titration curves. The J_{C-P} values have been determined for glyphosate as a function of pH and are used to help support the interpretation of the pH titration curves. The ^{31}P chemical shift of S3P while bound to EPSPS in the herbicidal dead-end ternary complex is reported with the ^{13}C, ^{15}N, and ^{31}P δs for glyphosate.

Materials and Methods

Materials. Isotopically labeled glyphosates, C-1 (^{13}C, 99%) and C-3 (^{13}C, 99%; ^{15}N, 99%), were gifts from Dr. Jacob Schaefer. [U-^{14}C]Shikimate was obtained from NEN and was used to make [U-^{14}C]S3P by the procedure described by Wibbenmeyer et al. (1988). [U-^{14}C]Glyphosate was obtained from the Monsanto Co. Deuterium oxide, ion free, was obtained from MSD Isotopes. (Aminomethyl)phosphonic acid (AMPA) (**3**) was obtained from Sigma and was of analytical grade. 4-Phosphonobutyric acid (**2**) was prepared by the method of Wasielewski and Antezak (1981). *N*-Methylglyphosate (**4**) [first described by Zyablikova et al. (1969)] was prepared by the method of Rogers (1986). *N*,*N*-Dimethylglyphosate (**5**) was a gift from Dr. L. Maier[2] of the Monsanto

Co. and was prepared by the method described for *N*-methylglyphosate by Zyablikova et al. (1969) except that *N*-methylglycine was used instead of glycine. The reaction involves heating *N*-methylglycine in caustic with 0.75 equiv of bis(chloromethyl)phosphinic acid in a sealed tube at 115 °C for 1.5 h with the product isolated by an acidic water workup. The mp was greater than 240 °C, and the proton spectrum in D_2O gave a singlet at 3.27 ppm (I = 6), a doublet at 3.68 ppm (I = 2), J_{H-P} = 12.8 Hz, and a singlet at 4.19 ppm (I = 2). All other general chemicals were of the highest purity available, and all gels, buffers, and resins were obtained from the Pharmacia Co. Protein was concentrated on Amicon YM10 membranes under argon pressure with final concentrating for the NMR samples done by Centricon 10 filtration (Amicon).

Enzyme Assays. Phosphate release was used for general rate assays using the Lanzetta (1979) dye method and is more accurately described by Padgette et al. (1987). Alternatively, a UV assay was developed that could be used during protein purification and routine assays. The assay follows the forward reaction by using the slight difference in UV absorbance in S3P and EPSP apparent at 244 nm when PEP is subtracted out by using a double-beam spectrophotometer (Kontron 820). The conditions for the assay were 1 mM PEP, 2 mM S3P, and initiation with EPSPS at room temperature. The blank cuvette contained only 1 mM PEP in the assay buffer [50 mM Hepes, pH 7.0, 86 mM KCl, 10% (v/v) glycerol, and 5 mM β-mercaptoethanol]. The time course was linear only for 30% of the reaction but follows the P_i release assay very closely. The extinction coefficient is approximately equal to that for the P_i assay when the P_i assay is done under these same conditions. However, the UV spectra of S3P and EPSP are quite sensitive to pH, and the assay is only useful for rapid qualitative determinations of activity.

Enzyme Purification. EPSPS is isolated from a strain of *E. coli* (pMON 6001) overexpressing EPSPS (Rogers et al., 1983). The purification is patterned originally after the procedure reported by Lewendon and Coggins (1983) but is substantially improved by the following procedures. The buffer used in all steps unless specifically noted is 0.1 M Tris-HCl, pH 7.5, 50 mM KCl, 10% (v/v) glycerol, 5 mM β-mercaptoethanol, and 1 mM EDTA. The cell paste (160 g) is melted in 500 mL of homogenate buffer (stock buffer plus 83 mg of ovomucoid trypsin inhibitor I, Sigma) and sonicated (Heat Systems Ultrasonics Inc. Model W 370, $^3/_4$-in. horn at full power) for 3-min pulses with stirring while bathed in a methanol/ice bath maintaining the temperature below 8 °C. The cell lysis is monitored by the UV activity assay to determine when activity release leveled off. The homogenate is then centrifuged (IEC M 25) 45 min at 51000*g* to pellet cell debris. The homogenate is scanned in the UV (Warburg & Christian, 1941) to determine the amount of protamine sulfate to add (Pittard & Ely, 1979) where 1 mL of 2% (w/v) protamine sulfate (Sigma) is added for every 140 mg of protein. This generally underestimates the amount of nucleic acids, so a 10% excess is added. The protamine/protein mixture is stirred 15 min and then centrifuged 15 min at 18000*g*. The protamine supernatant is then made 50% in $(NH_4)_2SO_4$, stirred 15 min, and then centrifuged 20 min at 51000*g*. The supernatant is then made 70% in $(NH_4)_2SO_4$ and stirred 20 min to bring down >98% of the enzyme, which is collected by centrifugation for 20 min at 51000*g*. The protein pellet is then redissolved in stock buffer plus 1.0 M $(NH_4)_2SO_4$ at approximately 12 mg/mL. The mixture can then be loaded directly onto a phenyl-Sepharose column (5 × 19 cm) equilibrated with the starting buffer [stock buffer plus 1.0 M $(NH_4)_2SO_4$]. The phenyl-Sepharose column is run at a flow rate of 7.5 mL/min, collecting 15-mL fractions for the linear gradient of 1.4 L of starting buffer against stock buffer. EPSPS elutes as a broad peak in fractions 50–115 just after the remaining nucleic acids. The fractions are pooled, precipitated with 70% $(NH_4)_2SO_4$, resuspended in 25 mM Tris-HCl, pH 7.5, 25 mM KCl, 10% (v/v) glycerol, 5 mM β-mercaptoethanol, and 1 mM EDTA at a protein concentration of 27 A_{280nm}OD units/mL, and dialyzed in 4.0 L of the same buffer overnight. The sample is then loaded directly onto a Q-Sepharose column (3 × 42 cm) equilibrated with the dialysis buffer at a flow rate of 20 mL/min. A linear gradient is run at the same flow rate between 25 and 300 mM KCl in the 25 mM Tris, pH 7.5, 10% glycerol, and 5 mM β-mercaptoethanol buffer by using 4.0 L of each. EPSPS elutes in fractions 46–66 (22 mL) in greater than 98% purity (specific activity was 90 IU/mg) as determined by silver staining on Lammeli gels (Dr. Richard Leimgruber, Monsanto Co. Physical Sciences Center). Overall recovery is approximately 80%, yielding in this case 1.75 g of enzyme. EPSPS for NMR samples is then dialyzed against 2.0 mM MOPS, pH 7.0, 25 mM KCl, 2% (v/v) glycerol, and 5 mM β-mercaptoethanol and frozen at −80 °C until needed. Protein assays are done by the method described by Zamenhoff (1957). The extinction coefficient for EPSPS is determined to be 0.77 A_{280nm}/mg by using the Zamenhoff assay and crystalline ribonuclease (Sigma) as a standard. NMR samples are concentrated to 6 mL by Amicon ultrafiltration and then further to 1.5 mL by twice centrifuging a Centricon 10 tube at 7000 rpm for 50 min.

NMR. ^{13}C spectra were acquired for the dead-end complex at 25 °C on a Varian XL 400 using Waltz decoupling during acquisition. The ^{15}N–^1H coupled spectrum was acquired at 25 °C. ^{31}P NMR spectra of the dead-end complex were acquired on a Varian XL 300 at 25 °C with Waltz decoupling. The titration curves for ^{31}P and ^{13}C were collected on an IBM AF 300 with Waltz decoupling during acquisition. All ^{13}C NMR spectra are referenced to glycerol in the dead-end complex samples with C-1 at 75.52 ppm. ^{31}P NMR spectra were referenced to 1 N phosphoric acid in D_2O (Cornelius et al., 1977) and collected with 1-Hz digitalization. ^{15}N spectra were referenced to 2.0 M $^{15}NH_4{}^{15}NO_3$ (99% labeled) in 1 N DCl in D_2O where NH_4^+ was assigned 0.0 ppm and phased negative while NO_3^{2-} was at 355 ppm downfield and phased positive (Witanowski, 1973).

A sample 50 mM in S3P and 1 mM EDTA in 30% D_2O was titrated between pH 3 and 11.0. Spectra were acquired on a Varian XL 300 at 25 °C with 512 transients. The ^{31}P δ vs pH titration curve for S3P was a plateau at 0.5 ppm below pH 4.0 and a plateau at 4.5 ppm above pH 8.0. The pK_a was calculated to be 6.41 by the method described in Table I.

Rapid Gel Filtration. The procedure was a slight modification of the original method described by Penefsky (1979). EPSPS samples (40 μg) were made up fresh prior to loading on a prespun 1-mL TB syringe with Sephadex G-50 superfine held in place with an Acro disk 13 (Gelman). The sample pairs, experimental/control, were immediately centrifuged on an IEC clinical centrifuge 4 min at speed 5 at room temperature. Samples were made up to 35 μL, mixed, loaded, and chased with 65 μL of buffer before spinning. The collected volume was measured by syringe. Protein recovery was de-

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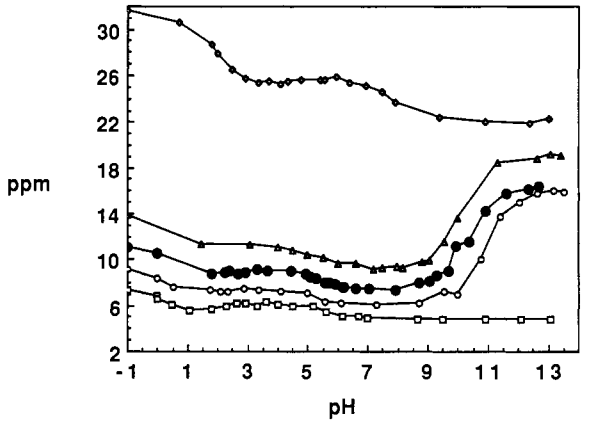


FIGURE 1: ^{31}P δ vs pH titration curves: (◇) 69 mM deazaglyphosate in 30% D_2O; (△) 430 mM (aminomethyl)phosphonate in 30% D_2O; (●) 50 mM glyphosate in 25% D_2O; (○) 73 mM *N*-methylglyphosate in 30% D_2O; (□) 64 mM *N,N*-dimethylglyphosate in 30% D_2O. These spectra were acquired on an IBM AF 300 with 512 transients at 25 °C. All samples were 1 mM in EDTA.

termined by area integration upon Mono Q HPLC (linear gradient in 25 mM Tris, pH 7.5, from 25 mM KCl to 300 mM in 20 min at 1 mL/min for the 5/5 column) using a standard curve and by recovered EPSPS activity. Routinely 80% of the protein applied was collected. Consequently, all calculations of equivalents isolated were corrected accordingly. Radioactivity was determined by mixing aliquots with 15 mL of Insta-Gel (Packard) and counting on a TM Analytic Mark III Model 6881 scintillation counter.

Four combinations of substrates and enzyme were evaluated for enzyme equivalents of radiolabeled ligand isolated by rapid gel filtration: (a) isolation of EPSPS S3P binary complex (E^{S3P}), using 66 μM EPSPS and 0.19 mM [^{14}C]S3P and yielding 0.02 equiv of S3P; (b) isolation of EPSPS–glyphosate binary complex, using 62 μM EPSPS and 0.77 mM [^{14}C]-glyphosate and yielding no radiolabel in the filtrate; (c) isolation of the dead-end ternary complex (E^{S3P}_{Glyph}), using 62 μM EPSPS, 0.75 mM [^{14}C]glyphosate, and 0.64 mM S3P and yielding 0.62 equiv of glyphosate; (d) isolation of the dead-end complex (E^{S3P}_{Glyph}), using 52 μM EPSPS, 0.53 mM glyphosate, and 0.25 mM [^{14}C]S3P and yielding 0.42 equiv of S3P.

Results and Discussion

Isolation of the Dead-End Ternary Complex by Rapid Gel Filtration. The relative stability of this herbicidal ternary dead-end complex is not generally appreciated. Studies meant to evaluate the stability of glyphosate on EPSPS using Penefsky-style (Penefsky, 1979) rapid gel filtration resulted in the isolation of 0.62 equiv of glyphosate/mol of EPSPS. Neither S3P nor glyphosate alone could be isolated by the technique. This result is the first tangible physical evidence for E^{S3P}_{Glyph}. EPSPS is still active upon dissociation of glyphosate, consistent with the covalent modification protection experiments where the dead-end complex protects EPSPS from thermal and oxidative denaturation (Kishore & Shah, 1988). The inherent stability of the dead-end ternary complex under these conditions suggested that the complex could be studied further by NMR.

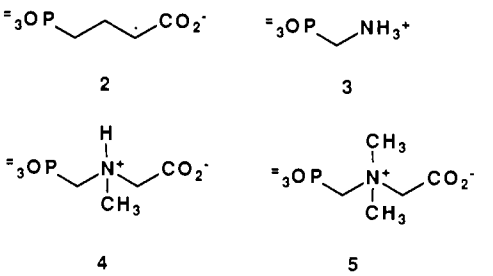
^{31}P NMR Chemical Shift Dependence for Glyphosate on pH. The chemical shift dependence on pH was determined for glyphosate (Figure 1) to determine the range of chemical shifts that might be observed upon binding to EPSPS. The ^{31}P δ titration curve for glyphosate [previously reported by Appleton et al. (1986), Sapozhnikova et al. (1986), and Smith et al. (1989)] was unusual in that the shielding trend observed

Table I: Summary of pK_a/$\Delta\delta$ from Figures 1, 4, and 6[a]

nucleus	sample	RPO_3H_2	RPO_3H^-	$R_2NH_2^+$
^{31}P	S3P	ND[b]	6.41/4.00	–
	AMPA	~0.5/2.54	5.29/1.87	10.09/9.85
	deaza	1.91/5.98	7.81/3.46	–
	N-methyl	~0.5/1.75	5.22/1.32	10.95/9.68
	N,N-dimethyl	~0.5/1.19	5.60/1.29	–
	glyphosate	~0.5/2.13	5.53/1.48	10.30/8.55
nucleus	sample	RCOOH	RPO_3H^-	$R_2NH_2^+$
^{13}C	glyphosate			
	C-1	2.24/2.73	–	9.84/8.77
	C-2	2.41/2.45	–	9.84/3.36
	C-3	–	5.43/1.74	9.80/2.11
^{15}N	glyphosate	–	–	10.15/10.83

[a] All pK_as were calculated by nonlinear regression using the equation described by Kurz and Frieden (1987). [b] ND, this titration was not determined.

for anion formation was reversed to deshielding upon deprotonation of the ammonium ion. These δ values and the explanation of them help to interpret the enzyme-induced $\Delta\delta$ for glyphosate. To assist the explanation, several other model compounds were also titrated to determine their ^{31}P δ vs pH (Figure 1). These results are summarized in Table I, where the pK_as derived from the curves are compared with the corresponding observed $\Delta\delta$ for glyphosate and the model compounds: 4-phosphonobutyrate (deazaglyphosate) (**2**); (aminomethy)phosphonate (AMPA) (**3**); *N*-methylglyphosate (**4**); and *N,N*-dimethylglyphosate (**5**).



Glyphosate's ^{31}P chemical shift upon titration to alkaline pH is correlated very closely with the ionization state of the amine. The pK_a of the amine determined in the ^{31}P δ vs pH titration curve is 10.3 (Table I) and is similar to values reported in the literature (Smith et al., 1989; Appleton et al., 1986; Sapozhnikova et al., 1986; Motekaitis et al., 1985; Madsen et al., 1978). To interpret the glyphosate titration curve, AMPA (**3**) was titrated to separate out potential interactions involving the carboxylate and the phosphonate anions. Figure 1 shows the profiles of AMPA and glyphosate to be nearly identical except for a slight upfield shift for the entire glyphosate curve. The carboxyl group in glyphosate is not a determining factor in the ^{31}P NMR δ vs pH titration curve at any pH.

This strongly suggests that the presence of the positive charge on the ammonium group is responsible for the large upfield shift under acidic and neutral conditions [first noted by Rueppel and Marvel (1976)]. Deazaglyphosate was titrated to test this hypothesis. The deaza analogue does not display the large upfield chemical shift in the acid and neutral pH region (Figure 1). Deazaglyphosate illustrates the shielding effect the ammonium ion of glyphosate has on the δ of the phosphonate; i.e., at alkaline pH, where the free base of the glyphosate amine is present, a δ = 16.4 is observed for glyphosate. This is near the δ value for deazaglyphosate at approximately 21 ppm under the same conditions. The comparison of glyphosate (**1**) to deazaglyphosate (**2**) and AMPA (**3**) unequivocally proves it is the nitrogen cation that is re-

sponsible for the dramatic upfield shift of the ^{31}P signal under neutral and acid conditions.

The ammonium protons in glyphosate could be expected to form intramolecular H-bonds with the phosphonate and carboxylate anions, and the formation of these two H-bonds may have an effect on the shielding observed in the titration curve. To evaluate the importance of the ammonium protons to the shielding observed in the glyphosate ^{31}P NMR titration curve, *N*-methyl- (**4**) and *N*,*N*-dimethylglyphosate (**5**) were titrated. The titration curves (Figure 1) show that *N*-methylglyphosate (**4**) like AMPA (**3**) is very similar to glyphosate, but shifted slightly more upfield than glyphosate. *N*,*N*-Dimethylglyphosate (**5**) is similar in the 0.0–9.0 region; however, since the cation still exists in basic solution, no downfield $\Delta\delta$ is observed. This confirms that the formation of the ammonium cation is responsible for the upfield $\Delta\delta$. A similar comparison has been made by Sapozhnikova et al. (1986) for glyphosate and *N*-methylglyphosate with the same results, but explained by the rehybridization of the amine and ammonium species. All four (aminomethyl)phosphonate derivatives are very similar with the following trends. The titration curves move progressively upfield with these δ values at pH −0.8: (**3**) 13.86; (**1**) 11.1; (**4**) 9.19; and (**5**) 7.36. This translates to an average increase in shielding of 1.95 ± 0.1 ppm for each additional alkyl substitution to the primary amine of **3**. Each curve was further shielded upon ionization of the first three oxygen acids (in the pH range from −0.8 to 7.5) with the following net $\Delta\delta$ for each: (**3**) 4.41; (**1**) 3.70; (**4**) 3.07; and (**5**) 2.48. The latter shielding trend, due to anion formation, is smaller in magnitude than the shielding observed for full ionization of deazaglyphosate ($\Delta\delta$ = 9.6). The shielding is also progressively diminished by substitution of alkyl groups for protons on the AMPA amine. If H-bond formation were the sole reason for the attenuation in the shielding effect on the phosphonate, as compared to deazaglyphosate, then alkyl substitution should have restored the magnitude of the observed shielding. Apparently the electrostatic effect of the ammonium ion involved in the ion pair is sufficient to decrease the electron density on the phosphonate oxygens, resulting in deshielding. Recently Baraniak and Frey (1988) showed that ion pairing in solution causes a measurable perturbation on the P–O bond order.

The glyphosate ^{31}P δ vs pH titration profile[3] in Figure 1 shows that in the pH regions below 0.0 and above 9.5 the same ^{31}P δ occur. These δ values cannot be explained by a similarity of the aqueous ionic environments; i.e., fully protonated glyphosate (+1) and fully ionized glyphosate (−3) are inherently in very different environments with very different overall charge. As an explanation, consider that these two ionic states may have similar conformations. Specifically, the $\Delta\delta$ observed for the ^{31}P NMR signals of glyphosate in the pH titration may be explained in terms of a common solution conformation for the (+1) and (−3) species with similar C–P–O bond angles for the glyphosate backbone.

Gorenstein (1984) has effectively argued that the $\Delta\delta$ in phosphates is correlated with the O–P–O bond angle. While phosphonates and phosphates mutually contain an O–P–O bond angle, the phosphonate chemical shift changes in an opposite fashion during titration with base. Empirically, the key factor for phosphonates is not the O–P–O bond angle, but the C–P–O bond angle. Hence, the ^{31}P NMR chemical shift moves upfield upon phosphonate ionization, while the O–P–O bond angle gets larger (as for phosphates) and concomitantly the C–P–O bond gets smaller.

We propose the similarity in ^{31}P δ for the two extreme pH regions can be explained by an equivalence of the average bond angles about the glyphosate backbone. The five ionic forms of glyphosate are labeled according to their charge and compiled in Table II with their calculated energies,[4] bond angles, and H-bond distances. These calculations for the five ionic forms of glyphosate (Table II) support the categorization of glyphosate into two general conformations. Glyphosate as different zwitterions in the pH range 0.0–9.5 can form intramolecular H-bonds, whereas glyphosate in the strongly acidic or strongly alkaline solution is not able to form effective intramolecular H-bonds. The ^{31}P δ vs pH titration curve for glyphosate can be divided into four pH regions, −1 to 1.5, 1.5–4, 4–8, and 8–14, containing each of the ionizations for glyphosate with pK_as of ~0.5, 2.24, 5.53, and 10.30, respectively, which compare very well to 0.492, 2.229, 5.460, and 10.142, the values obtained by Smith et al. (1989) using ^{31}P NMR.

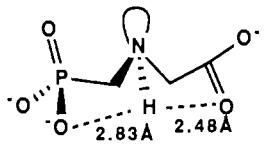
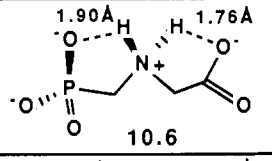
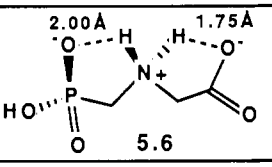
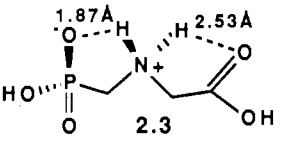
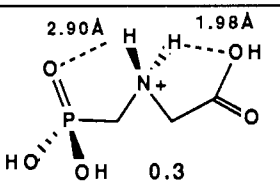
Under strongly acidic conditions the net positive species of glyphosate is produced (+1). The phosphonate and carboxyl are fully protonated, and ion–ion repulsive forces between oxygens are minimized; the C–P–O and C–C–O bond angles should be nearly ideal. This is supported by the bond angles predicted from the calculations in Table II (+ refers to bond angle degrees predicted larger than ideal and − to predictions smaller than ideal). There are dipole–dipole repulsion forces between the protonated oxygens and weak electrostatic ion–dipole attraction forces between the ammonium ion and the C=O and P=O dipoles. However, these latter forces are respectively weaker and not sufficient to pinch the C–P–O bond angle.

As the pH becomes more basic, the very acidic ionization of the first phosphonate oxygen (pK_a ~ 0.5) allows the formation of an internal H-bond for the neutral species (0). It is important to realize that the cationic charge of the ammonium ion is not on the nitrogen atom (Clementi, 1967), but is distributed over the two H's and the adjacent carbons. This distribution of positive charge on the carbon backbone is discussed for amino acids by Horsely and Sternlicht (Horsely & Sternlicht, 1968; Horsely et al., 1970). The formation of this H-bond seems to result in a substantial stabilization of the 0 species as compared to the +1 species as the calculated minimum energy goes from −10.95 to −39.25 kcal/mol (Table II). The C–P–O bond angle is predicted to be pinched 7.6° with a much improved H-bond formed compared to that of the +1 structure. This internal H-bond provides the overall favorable energy, although the importance of this effect will be attenuated by solvation in a polar medium. The crystal structures for the 0 species (Sheldrick & Morr, 1981; Knuuttila & Knuuttilla, 1979; Shkolnikova et al., 1982) show only a few differences when compared to the predicted MacroModel structure. MacroModel seems to have pinched the N–C–P bond 10° too much, but in a compensating way underestimated the distortion of the phosphonate O–P–O bond angles (106.0°, 118.7°, and 111.7° for X-ray vs 107.5°, 111.6°, and 108.9° for MacroModel, respectively). Both structures show a phosphonate oxygen H-bonded to the ammonium proton with the X-ray $PO^-\cdots H$ distance of 2.21 Å and a P–O–H angle of 89.4° with MacroModel predicting a $PO^-\cdots H$ distance of 1.87 Å and a P–O–H angle of 90.6°. The ^{31}P NMR signal is shielded upon the first ionization of the phosphonate, consistent

[3] These data correlate very well with unpublished data obtained by Dr. J. E. Franz and C. Mamer, Monsanto Agricultural Co., private communication.

[4] Note that the minimized energies are not directly comparable due to the addition of a proton to each successive species; i.e., the chemical formulas are not isomeric.

Table II: Comparison of MM2 Bond Angles for Glyphosate Species[a]

Species		Bond angles[b]					Energy
		C-P-O	P-C-N	C-N-C	N-C-C	C-C-O	
Charge	pKa	110.0	110.0	108.6	110.7	117.0	Kcal/mole
-3		+0.6	+3.6	+4.8[c]	+0.5	+1.8	+12.89
-2	 10.6	-7.9	-8.6	+7.8	-3.9	-6.8	-31.03
-1	 5.6	-6.3	-8.0	+7.7	-4.0	-6.4	-39.90
0	 2.3	-7.6	-8.5	+6.0	-0.5	+1.8[d]	-39.25
+1	 0.3	-0.6	+3.5	+6.3	-1.2	+1.3[e]	-10.95

[a] All calculations were performed by using the MacroModel program (version 1.5, W. C. Still, Columbia University). The MM2 force field within MacroModel was used in all calculations. Block diagonal Newton Raphson minimization was implemented along with terminal atom movement. All minimized structures had a first derivative RMS of less than 0.008 kJ/Å. Bond lengths and distanes reported are from minimized structures. Bond angles are from minimized structures and recorded as the difference between the calculated ideal bond angle and the calculated actual bond angle when force constants are applied. Each individual ideal bond angle is listed under the respective label. [b] Bond angles are in degrees and represent the difference from the ideal angle for each column. [c] The ideal bond angle was 107.7°. [d] The ideal bond angle for the H-bonded C=O was 122.5°. [e] The ideal bond angle for the H-bonded OH was 107.1°.

with increased electron density at phosphorus. The upfield $\Delta\delta$ = 2.13 seems to be attenuated by the neighboring ammonium group since for deazaglyphosate (Figure 1) the $\Delta\delta$ (5.98) for the first phosphonate ionization is more than twice as large. Experimental support for the formation of this H-bond can be found in the infrared spectrum recorded by Shoval and Yariv (1981). The infrared spectrum of glyphosate contains absorptions attributed to P=O that are somewhat weaker for the K^+ salt than for the HCl salt, however, not weak enough to account for equalization of the oxygens at phosphorus. Therefore, one of the oxygens is thought to be more strongly H-bonded than the others. Shoval and Yariv also noted that the absorptions for the NH_2^+ group were perturbed in the K^+ salt as compared to the HCl salt, indicative of H-bond formation, probably to the phosphonate oxygen anion.

Ionization of the carboxylate ion of the 0 species at pK_a = 2.24 forms −1 glyphosate, the most stable zwitterion, as predicted by calculations (but not the predominant form at pH 7.0). The −1 species is predicted to have an optimized conformation due to the formation of two H-bonds forming a spirocyclic-like system. The two predicted internal H-bonds have reasonable angles at PO–H–N (122.4°) and CO–H–N (115.5°) with H-bond lengths of 2.00 and 1.75 Å, respectively, which allow these two interactions to substantially stabilize the calculated spirocyclic structure. This structure has the same problems as the 0 species discussed above when compared to the X-ray crystal structure for the −1 species reported by Smith et al. (1989). The ^{31}P NMR signal is virtually unaffected by the carboxyl ionization when the glyphosate curve is compared to AMPA for the same pH region (Figure 1).

Ionization of the second phosphonate oxygen, pK_a = 5.53, is visualized in Figure 1 by an upfield ^{31}P $\Delta\delta$ = 1.48 (Table I) upon formation of the −2 species, the predominant form at pH 7.0. This $\Delta\delta$ is attenuated by the ammonium moiety in comparison to deazaglyphosate, which has a $\Delta\delta$ = 3.46. Again the increased electron density on phosphorus is consistent with shielding and correlates to pinching of the C–P–O bond angle. The increase in negative charge at the phosphonate shortens the calculated PO–H H-bond length to 1.9 Å, while the carboxylate H-bond length is unaffected. The minimized energies in Table II show that the −2 species is overall destabilized by the formation of the third anion primarily through ion–ion repulsive forces between the phosphonate oxygens. The 2 pH unit acidic pK_a shift measured for the phosphonate of glyphosate relative to deazaglyphosate is a reflection of the α-amino effect, typical of amino acids (Gutfreund, 1972). The X-ray crystal structure reported by Smith et al. (1989) is very similar to the MacroModel structure except for the same differences pointed out for the 0 and −1 species above and with

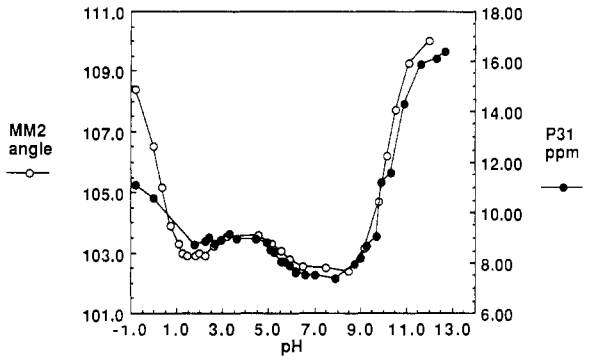


FIGURE 2: Comparison of the ^{31}P NMR δ of glyphosate to the C–P–O bond angle as derived from the minimized structures in Table II.

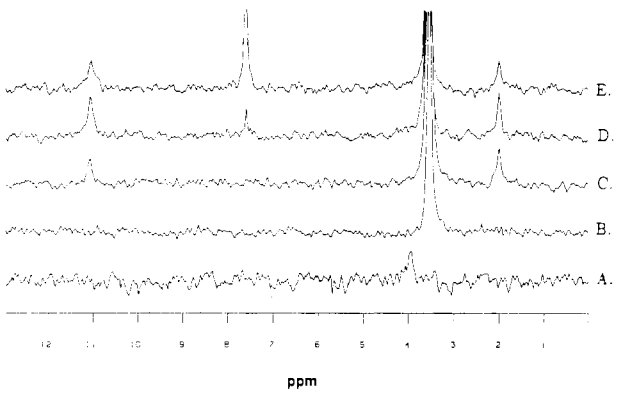


FIGURE 3: Titration of EPSP synthase with S3P and glyphosate: (A) 1 mM EPSPS in 2 mM MOPS, 5 mM β-mercaptoethanol (pH 6.60, pD 7.00), 0.01 mM EDTA in D_2O with 0.8 mM S3P; (B) 1 mM EPSPS as in (A) with 10 mM S3P; (C) 1 mM EPSPS as in (A) with 10 mM S3P and 0.8 M glyphosate; (D) 1 mM EPSPS as in (A) with 10 mM S3P and 1.6 mM glyphosate; (E) 1 mM EPSPS as in (A) with 10 mM S3P and 4 mM glyphosate. These spectra were acquired on a Varian XL 300 at 25 °C with a 5 mM probe. The spectra in (A) and (B) were 512 transients and (C), (D), and (E) with 2000 scans. The proton-coupled spectrum (data not shown) gave a doublet with J_{P-H} = 8.8 Hz for S3P and a triplet with J_{P-H} = 11.6 Hz for glyphosate. The signals at 11.0 and 1.99 were not resolved at 25 °C; however, the 1.99 signal could be resolved into a doublet, J_{P-H} = 6.6 Hz, at 45 °C, identifying that signal as enzyme-bound S3P.

the backbone twisted due to different torsional angles.

Above pH 9.0 an 8.9 ppm downfield ^{31}P NMR $\Delta\delta$ migration is observed as the ammonium ion is deprotonated to form the −3 species. This deshielding might be explained by the disappearance of the cation with its electric field effect or by changes in conformation affecting the C–P–O bond angle (but consider that the two effects may not be separable). Heubel and Popov (1979) show in a ^{31}P δ vs pH titration for phosphonoacetic acid that ionization of the carboxylate causes a similar downfield shift for the ^{31}P NMR signal. These two cases are similar in that deshielding is observed upon formation of electron density, deprotonation of ammonium ion for glyphosate, and ionization of carboxylic acid for phosphonoacetic acid. The deshielding is correlated to an "opening" of the C–P–O bond angle resulting from the repulsion forces between the lone pair electrons in glyphosate or carboxylate anion in phosphonoacetic acid and their phosphonate oxygen anions. In glyphosate this repulsive dipole–ion force has a substantially greater effect on the C–P–O bond angle than the ionic repulsion between phosphonate oxygen anions; compare the C–P–O bond angles for structures −3 and −2.

The minimized structures for the −3 and +1 species are remarkably similar, with the +1 structure being stabilized slightly by the weak H-bonds of the protonated oxygens. The two charged groups, PO_3^{2-} and CO_2^-, in the −3 structure now calculate close to ideal bond angles (Table II, C–P–O and C–C–O) when the H-bonding opportunity is removed. In addition, these latter two structures are also distinctly different from the 0, −1, and −2 structures that have their own general similarities. These data are supported by the calculated minimized structures and suggest how the same average solution conformation for glyphosate can exist at two very different pHs.

The hypothesis that the C–P–O bond angle is correlated to the ^{31}P NMR δ is tested in Figure 2. The C–P–O bond angle for each of the five glyphosate charged species is plotted against pH by utilizing the pK_as in Table I. The two curves, C–P–O bond angle vs pH and ^{31}P δ vs pH, are strikingly similar except for the +1 species. Therefore, the phosphonate ^{31}P NMR δ does successfully predict the C–P–O bond angle. MacroModel probably does overestimate the attractive forces of charge and the resulting H-bonding effect, hence predicting smaller angles, as discussed above when compared to the X-ray results. However, the structures predicted by MacroModel for glyphosate throughout the pH range do seem to correlate to the ^{31}P NMR data.

These observations are made for compounds 1–5 from their pH titration curves in Figure 1 with respect to the ^{31}P NMR δ: (1) The positive charge on nitrogen exerts the largest effect on the phosphonate chemical shift of glyphosate in the entire pH range. (2) While fully protonated at pH −0.8, the phosphonate NMR signal is shielded by progressive alkyl substitution at the α nitrogen with an average shielding of 1.95 ± 0.1 ppm per alkyl group. (3) The average shielding for the three glyphosates (**1**, **4**, and **5**) vs deazaglyphosate (**2**) during the titration of the second phosphonate proton is rather constant at $\Delta\delta$ = 1.36 vs $\Delta\delta$ = 3.16, respectively. The average total shielding due to titration of all three oxygen anions for the three glyphosates vs deazaglyphosate is $\Delta\delta$ = 3.1 vs $\Delta\delta$ = 9.6. (4) The second pK_a of the phosphonate for compounds **1**, **3**, **4**, and **5** is about 5.40, in contrast to the pK_a of deazaglyphosate (**2**) at 7.81. The amine pK_as follow a monotonic trend in increasing basicity from primary to tertiary amine as determined from the ^{31}P NMR data (Table I). The general upfield migration of the ^{31}P NMR δ with increasing basicity in the titration curves in Figure 1 is opposite to the downfield trend observed by ^{15}N NMR in going from primary ammonium ion to the quaternary ammonium salt. Literature ^{15}N δ values show that quaternary ammonium salts are furthest downfield in an alkylamine series [see Webb (1986)].

^{31}P NMR of S3P Bound to EPSPS, Binary and Dead-End Complexes. The ^{31}P NMR of E^{S3P} under conditions where 96% of the S3P is bound shows a 0.54 ppm downfield shift relative to free S3P (Figure 3A). The chemical shift of S3P (3.9 ppm) bound in the E^{S3P} complex corresponds to a pH of 7.15, a slightly more basic δ than free S3P at 3.54 ppm in the absence of EPSPS. This is modeled by increased ionization of S3P if a pH titration curve is used to explain the δ or an increased O–P–O bond angle using Gorenstein's argument. As an excess of S3P (Figure 3B) is added, the signal moves back upfield, consistent with the δ of free S3P (3.54, J_{H-P} = 8.7 Hz) at this pH. Hence, the δ for S3P is dependent on the percent of the population of bound vs free (Feeny et al., 1979) and is consistent with the reported exchange rates (K_d = 7 μM and k_{off} = 4500 s^{-1}) measured by Anderson et al. (1988b). The addition of glyphosate as shown in Figure 3C–E results in a new S3P resonance at 2.0 ppm, corresponding to pH 6.15 for the δ of free S3P as a function of pH. The proton-coupled ^{31}P spectrum shows the 2.0 ppm signal to be a doublet (J_{H-P} = 6.6 Hz, data not shown) confirming its origin as enzyme-bound S3P. The $\Delta\delta$ of 1.94 upfield between binary S3P

(Figure 3A) and S3P bound in the dead-end complex (Figure 3C) can be explained by net protonation of the shikimate 3-phosphate upon glyphosate binding.

An extension of Gorenstein's idea of relating the O–P–O bond angle in phosphate esters to chemical shift is to consider that the P–O–C bond angle is also important in determining the chemical shift for phosphate esters. Since binding of small charged molecules is energetically dependent, to a large degree, on correctly positioning the charged groups for binding, then the COO^- and OPO_3^{2-} groups may be considered as firm anchors for the rest of the S3P. Constraining these two binding contacts can dictate a particular S3P ring conformation. The subsequent binding of glyphosate could alter that orientation by inducing an enzyme conformational change affecting the relative positions of the COO^- and OPO_3^{2-} groups, thus changing the P–O–C bond angle to the S3P ring. An enzyme conformational change is implicated by intrinsic fluorescence studies (Anderson et al., 1988a) upon glyphosate binding.

^{31}P NMR of Glyphosate in the Dead-End Complex. Addition of less than 1 equiv of glyphosate to the preformed binary complex (Figure 3C) results in the formation of two new resonances: a downfield signal at 11.0 ppm, corresponding to bound glyphosate, and a matching signal upfield at 2.0 ppm, identified as protein-bound S3P. As more glyphosate (Figure 3D) and then finally an excess of glyphosate is added (Figure 3E), a fourth signal corresponding to free glyphosate appears at 7.5 ppm (J_{H-P} = 11.5 Hz). These spectra show that the rate of exchange for glyphosate (K_i = 0.16 μM with k_{off} = 0.12 s^{-1}; Anderson et al., 1988a) is slow on the NMR time scale, and consequently, a discrete enzyme-bound species can be observed.

The binding of glyphosate in E^{S3P}_{Glyph} induces a downfield $\Delta\delta$ = 3.5 for the glyphosate ^{31}P NMR signal. The bound glyphosate ^{31}P NMR signal at 11 ppm intersects the glyphosate ^{31}P δ vs pH titration curve at two points (pH −0.8 and 10.1, Figure 1). The downfield δ perturbation induced by the enzyme binding site on the phosphonate ^{31}P NMR signal could be explained by several different effects or even the sum of several different effects: environmental hydrophobic effects, protonation, substitution of cation, and C–P–O bond angle.

First, consider the nonspecific hydrophobic protein environmental effect involved in the neutralization of a charged species. The movement of a solvent-stabilized anion into a nonaqueous environment will substantially destabilize the anion or increase basicity. The compensating effect on the protein is that the solvent-hydrated cation (usually a lysine or arginine in nonmetallic requiring systems) is also destabilized in the hydrophobic pocket and made more acidic. Simplistically, then, if a hypothetical lysine residue is destabilized enough in a hydrophobic pocket to lower the pK_a from 11 to 9 [and there are documented cases of much larger perturbations in lysine pK_as; Westheimer and Schmidt (1971) report a pK_a = 5.9] and the same hydrophobic site destabilizes the phosphonate anion enough to raise the pK_a from 5.4 to 7.4, then the neutralization becomes possible. However, only full protonation will explain all of the $\Delta\delta$ observed on the basis of the titration curve in Figure 1 since a pH environment of −0.8 results in a ^{31}P NMR signal shifted downfield to 11 ppm. Complete protonation at oxygen seems improbable since the proton donated is likely to be still coordinated with the proton donor (H-bonded), necessarily decreasing the strength of the new neutralizing H-bond and the net protonation state.

A deshielding effect arising from simply substituting cations will not explain the entire 3.5 ppm downfield shift. For example, glyphosate is predicted to form two tight intramolecular H-bonds between the phosphonate oxygen anion, the carboxyl oxygen anion, and the two ammonium protons (see Table II for calculated H-bond lengths). The internal secondary amine then serves as a counterion for the two anionic groups. This intramolecular H-bond formation occurring concomitant with oxygen anion formation is characterized by shielding of the ^{31}P NMR signal. In contrast, the formation of an external H-bond for these same oxygen anions to the protein, via a similar ammonium cation (lysine or arginine), seems unlikely to cause a net 3.5 ppm downfield $\Delta\delta$ as observed in Figure 3 for glyphosate in E^{S3P}_{Glyph}.

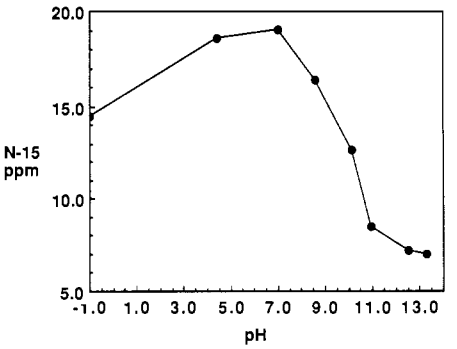


FIGURE 4: ^{15}N chemical shift vs pH titration curve for glyphosate. One 46 mM glyphosate sample in 10% D_2O was used for all of these spectra. The indicated pHs were obtained by titration with 5 N NaOH or 5 N HCl as needed. Spectra were acquired on a Varian XL 400 at 25 °C with 2000 transients.

The largest perturbation on the chemical shift may be attributed to bond angle changes associated with conformational changes in the glyphosate backbone induced by binding to EPSPS. The protein can offer a minimum of five intermolecular ionic H-bonds (three for oxygen anions and two for the ammonium protons) to glyphosate upon ternary complex formation. Hence, upon glyphosate binding, a net energy stabilization of three ionic H-bonds will dictate the glyphosate conformation. Therefore, if intramolecular H-bond formation pinches the C–P–O bond angle and results in an upfield shift of the phosphonate signal, then just as removal of those intramolecular H-bonds relaxes the backbone and results in a downfield $\Delta\delta$, so does binding to the enzyme relax those same angles since a downfield shift is observed upon binding. Hence, the conformation of glyphosate bound in the ternary complex is similar to the average solution structure at either pH 10.1 or −0.8.

It seems plausible then for the downfield $\Delta\delta$ observed for the glyphosate phosphonate upon dead-end complex formation to depend largely on the conformation assumed by glyphosate due to the constraints enforced by EPSPS. These conformational constraints arise mainly from the energy gained by charge neutralization. Alternatively, glyphosate could be bound as a species approximately 48% in the free base form, or the average species present at pH 10.1. However, the ^{15}N NMR results discussed below do not support this suggestion.

^{15}N NMR of Glyphosate vs pH. The general sensitivity of the ^{15}N nucleus to environment and bond hybridization is reflected in the very wide range of chemical shifts for nitrogen-containing compounds (Witanowski, 1973). The ^{15}N NMR of isotopically labeled glyphosate bound in the dead-end complex should reveal the protonation state of the ammonium ion. An interpretation of the experimental result for the ^{15}N δ perturbation in the dead-end complex requires an understanding of the possible range of chemical shifts for glyphosate and an explanation for the $\Delta\delta$ trends observed during a pH titration. A comparison of the ^{15}N NMR titration curves for glyphosate (Figure 4) and that of glycine reported by Leipert

and Noggle (1975) shows the two curves to be very similar for the entire pH range (chemical shift reference notwithstanding). Leipert and Noggle pointed out that ionization of the carboxyl of glycine (in going from pH 0.5 to 6.3) deshields the ^{15}N signal of glycine ($\Delta\delta$ = 1.8) and noted this deshielding was consistent with H-bonding. This deshielding associated with carboxylate ionization in glycine was removed in the ^{15}N NMR δ vs pH titration of ethyl glycinate, where the δ was constant for the pH range 0.5–6.6, by Cooper et al. (1973). A more general ^{15}N NMR study of several different amino acids by Blomberg et al. (1976) also confirms these findings. They determined the pH dependence for proton exchange on nitrogen and calculated their respective energies of activation for breaking the N–H bond. The large values measured (8–12 kcal/mol) are substantially larger than the typical range of 2–3 kcal/mol for H-bond energies (Fersht, 1987; Street et al., 1986).

The relative importance of the intramolecular H-bond at neutral pH formed by the phosphonate of glyphosate with the ammonium group is reflected in the energies for individual charged species in Table II. In the acidic pH region we argued above that protonation of the phosphonate weakened the H-bond and removed the electrostatic ion–ion attractive force that resulted in the downfield migration of the ^{31}P δ, postulating an increase in the C–P–O bond angle. A similar argument will also explain the upfield $\Delta\delta$ observed for ^{15}N if there is a correlation of the C–N–C bond angle with chemical shift. The primary difference in the C–N–C bond angle (Table II) is upon protonation. The sp^3 hybridized amine in the −3 species is pyramidally shaped, whereas the ammonium ion (species −2, −1, 0, +1), also sp^3 hybridized, is more perfectly tetrahedral. In addition, the formation of two H-bonds as in structures −2 and −1 is predicted to have the larger C–N–C bond angle, possibly due to compensations in the backbone for the formation of the spirocyclic intramolecular H-bond system. The two species with one H-bond have a slightly less perturbed C–N–C bond angle. A dependence of ^{15}N chemical shifts on the C–N–C bond angle can be obtained from tables of chemical shifts of compounds compiled by Levy and Lichter (1979) and more specifically from data presented by Lichter and Roberts (1972). Lichter and Roberts report that azetidine (25.3 ppm) is more upfield than pyrrolidine (38.2 ppm), which is similar to piperidine (39.6 ppm), morpholine (31.6 ppm), and piperazine (35.2 ppm). More data are required (under aqueous conditions) to support a general correlation of C–N–C bond angles to chemical shifts, but clearly the larger angles of the six-membered rings are shifted downfield. Correlations to δ have been made between N–H bond lengths and geometry in ab initio calculations by Ratcliffe et al. (1983).

^{15}N NMR of the Dead-End Ternary Complex. Figure 5 shows the fully coupled ^{15}N spectrum of E^{S3P}_{Glyph} formed with ^{15}N-labeled glyphosate. Two signals are seen: a broad signal at 23.4 ppm corresponding to EPSPS-bound glyphosate and a sharp signal at 18.4 ppm corresponding to free glyphosate. A comparison of the protein-bound glyphosate ^{15}N δ with the pH titration curve in Figure 4 shows the bound glyphosate ^{15}N resonance to be deshielded outside the expected chemical shift range. Thus, the glyphosate ^{15}N signal at 23.4 ppm, when bound in E^{S3P}_{Glyph}, requires that glyphosate be completely protonated at nitrogen.

An explanation for the excess 5 ppm deshielding observed for the ^{15}N signal upon binding E^{S3P} could be based on general protonation, ionic or hydrophobic effects, and possibly the C–N–C bond angle. First consider the upfield shift due to an EPSPS environment of pH 10.1 for glyphosate's amine.

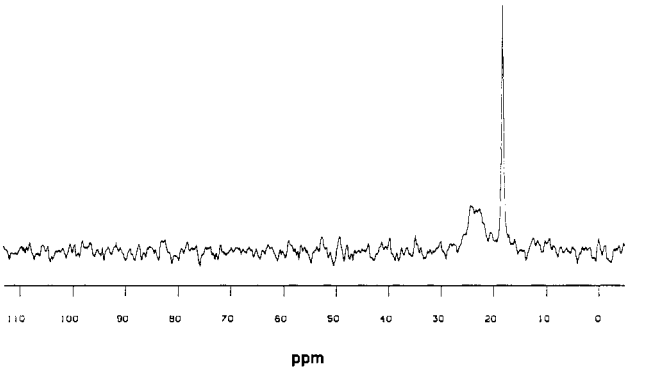


FIGURE 5: ^{15}N NMR of the dead-end complex (E^{S3P}_{Glyph}). The sample contained 1.6 mM EPSPS, 25 mM HEPES, 25 mM KCl, 2% glycerol, 5 mM β-mercaptoethanol, 0.01 mM EDTA, 2 mM ^{15}N (99%) glyphosate, and 10 mM S3P at pH 7.0 in 10% D_2O. The spectrum was acquired at 25 °C on a Varian XL 400 for 56 000 transients.

There would be a 7.2 ppm (18.4 − 11.2) upfield shift between the observed signal for glyphosate free in solution at pH 7.0 and the δ paired with pH 10.1 (indicated by the ^{31}P and ^{13}C signals for protein-bound glyphosate). This partially deprotonated nitrogen (pK_a = 10.15) must then be shifted downfield 12.2 ppm (11.2–23.4) to 23.4 ppm to compensate for the shielding due to deprotonation by the pH 10.1 environment to result in the net downfield shift observed. Since there is a general downfield shift of nitrogen signals when moving to higher dielectric environments, this glyphosate species must be bound in a pocket with a much higher dielectric than water to result in such a large downfield shift. The magnitude of the shift required is unlikely in view of the results reported by Duthaler and Roberts (1978). They reported for a series of primary, secondary, and tertiary amines a $\Delta\delta$ = 3.6 ppm downfield for secondary amines upon formation of the HCl salt in methanol and only an upfield shift of 0.7 ppm upon going from methanol to benzene with the free base. The trend in the titration curve upon protonation of glyphosate free base is in the predicted direction, i.e., reduced electron shielding upon protonation causing a downfield shift. Kricheldorf (1979) studied amino acids by ^{15}N NMR in different acidic aqueous solutions to show that very small upfield $\Delta\delta$ are observed (less than 1.5 ppm) by changing the counteranion in strong acid solutions. These $\Delta\delta$ were correlated to hydration and solvent effects, but the main effect for amino acids was due to interactions of α substituents with the solvation shell of the ammonium and carboxyl groups. Hence, solvent-type effects will not explain the observed $\Delta\delta$ for the ^{15}N signal.

The EPSPS-induced $\Delta\delta$ for the glyphosate ^{15}N signal can be correlated to the C–N–C bond angle. For example, in alkaline solution, the lone pair is present on nitrogen and, although sp^3 hybridized, is pyramidally shaped due to the repulsion of the lone pair for the other orbitals. This results in the smallest C–N–C bond angle for the series in Table II. If the nitrogen is protonated, forming the ammonium ion, the repulsive dipole–dipole force is removed and a more nearly tetrahedral arrangement predominates for the rest of the species (−2 to +1). Hence, if a decrease in the C–N–C bond angle is correlated with an upfield shift, then the downfield shift observed for glyphosate bound in E^{S3P}_{Glyph} could be related to an increase in the C–N–C bond angle.

^{13}C NMR δ vs pH for Glyphosate. Carboxyl groups are also sensitive to environment and ionization state, and ^{13}C NMR provides a method to characterize the carboxyl group of glyphosate while bound to EPSPS. A number of empirical equations have been developed for predicting ^{13}C chemical shifts, and several have been developed specifically for amino

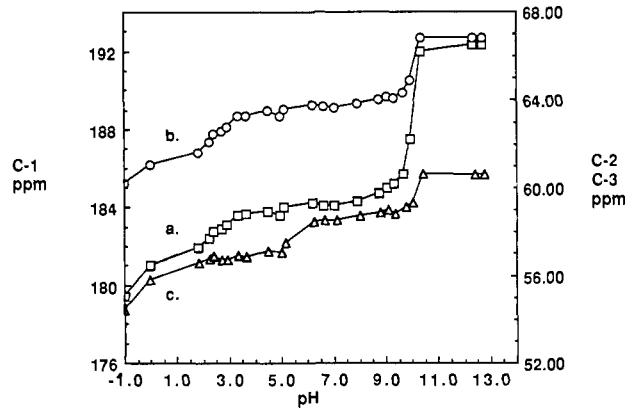


FIGURE 6: ^{13}C chemical shift vs pH titration curve for glyphosate. The same sample used for ^{31}P in Figure 1 was used. The titration curves for C-1 (□), C-2 (○), and C-3 (△) are compared as a function of pH. These spectra were acquired on an IBM AF 300 with 10000 transients at 25 °C.

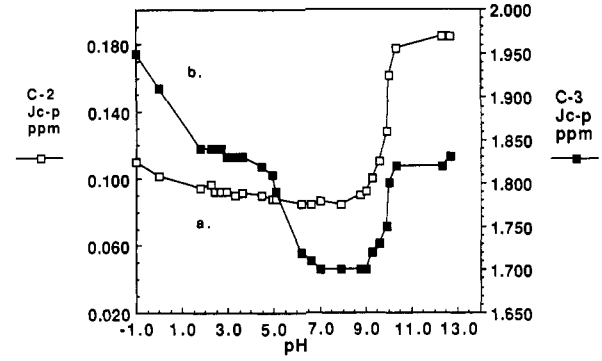


FIGURE 7: (a, □) ^{13}C coupling constants, $^3J_{C_2-P}$ vs pH for glyphosate. This curve reports only the ammonium pK_a = 9.84, which compares very well to the values reported by all three carbons in Table I. The same sample was used for ^{31}P NMR in Figure 1. (b, ■) ^{13}C coupling constants, J_{C_3-P} vs pH for glyphosate. The curve reports three pK_as. The first pK_a is the first phosphonate proton at approximately pH 0.5. The second pK_a = 5.52 compares very well to the pK_a = 5.53 in Table I for the glyphosate phosphonate. The last pK_a = 9.84 compares very well to the ammonium pK_as reported by all three carbons.

acids (Suprenant et al., 1980; Rabenstein & Sayer, 1976; MacDonald et al., 1976; Batchelor, 1975; Batchelor et al., 1975; Horsely & Sternlicht, 1968; Horsley et al., 1970). Data for glycine and other amino acids in aqueous solutions at different pHs are reported, and most amino acids by ^{13}C NMR have a constant deshielding of $\Delta\delta$ = 2.4 for the ionization of the COOH group. Glyphosate has a ^{13}C $\Delta\delta$ = 2.73 for this ionization centered around a pK_a = 2.24 (Table I). The magnitudes of the pK_as for a variety of carboxylic acids have been correlated to the $\Delta\delta$ by London (1980), who noted that deviations to this empirical observation were a measure of the extent and nature of environmental perturbations of the carboxyl group. London also noted than an explanation for the pK_a titration δ shift correlation could possibly be based on electron delocalization. Another empirical equation has been developed by Borthwick (1980) for carboxylic acid on the basis of their bond lengths and angles. Possibly the two empirical equations can be related and form a relationship correlating C–C–O bond angle to δ.

The large shielding of the carboxyl group in glyphosate upon protonation of the amine in the ^{13}C δ vs pH profile in Figure 6 is very characteristic for α-aminocarboxylic acids. In every pH titration of the α-amino group in α-aminocarboxylic acids that is followed by ^{13}C NMR, it is observed that protonation of the α-amino group results in a large shielding $\Delta\delta$ for the carboxyl group (Suprenant et al., 1980). The effect is generally ascribed to the linear electric field shift (LEFS) in explaining carbon data (Batchelor, 1975; Batchelor et al., 1975) where these effects dominate the changes in chemical shift. This general phenomenon for α-amino acids to be shielded upon ammonium ion formation may be due to the strong H-bond formed by the carboxylate anion with the α-ammonium group as shown in Table II for glyphosate. In Table II the C–C–O bond angle is very much dependent on H-bonding ability. Calculated structures −2 and −1 both show strong H-bonds for the carboxyl group as measured in the H-bonding length as compared to the 0 and +1 structures where little if any H-bond is possible. The conclusion could be that a decrease in the C–C–O bond angle results in shielding.

The ^{13}C chemical shift dependence on pH for the C-1, C-2, and C-3 of glyphosate all reflect the same pK_a = 9.84 for the amine even though each carbon is affected differently; i.e., their overall $\Delta\delta$ for this pK_a is different (Table I). These carbon spectra were acquired on exactly the same samples as used in the ^{31}P titrations. However, the ^{31}P δ vs pH curve reports an amine pK_a = 10.30. Why these two ionic groups, apparently both involved in H-bonding to the ammonium ion, report the ionization state differently by 0.46 ppm is intriguing and remains unexplained. In addition, these two values nearly average to the result obtained by ^{15}N NMR (Table II). Finally, the C-2 carbon reflects the pK_a of the carboxylate at 2.39 compared to the observed value of 2.24 for the carboxylate carbon. The C-3 carbon reflects a pK_a = 5.43 for the phosphonate (Figure 6b), while the ^{31}P δ vs pH curve gives a pK_a = 5.53 (Figure 1).

The pH effect is measured on J_{C_3-P} and $^3J_{C_2-P}$ for the same glyphosate samples used in the ^{31}P and ^{13}C titration curves in Figure 1. The $^3J_{C_2-P}$ data in Figure 7a generally feature only the effect of the ammonium ionization reporting a pK_a = 9.85. The J_{C_3-P} data in Figure 7b, however, reflect both phosphonate ionizations and the ammonium ionization. Three bond-coupling constants are generally sought as a means to define dihedral angles and hence conformations by using the Karplus equation. London et al. (1978) studied the ^{13}C–^{13}C coupling constants for aspartic and glutamic acids with respect to pH and reported a $^3J_{C_2-C_5}$ δ vs pH for glutamic acids that is very much like the pH dependence for the J_{C_3-P} curve shown in Figure 7. London's titration curve for glutamic acid also reports the pK_a of the γ-carboxyl and the α-amino groups. Huebel and Popov (1979) completed a study of phosphonoacetic acid focused on a variety of physical methods to determine the pK_as. They noted that the magnitude of the J_{C-P} values varied with pH in the same way as the ^{31}P NMR δ and also reflected the pK_as. The effect of ionizable groups on the coupling constant may be attributed strictly to an electronic effect; however, the formation of charged groups in species that can form intramolecular H-bonds will affect the conformation. Therefore, the electronic effect may be manifest in the traditional conformational effect on the coupling constant for species with ionizable groups that can form internal H-bonds.

^{13}C NMR of Glyphosate in E^{S3P}_{Glyph}. Glyphosate enriched with ^{13}C (99%) at C-1 was examined in E^{S3P}_{Glyph}. Figure 8 contains the ^1H-decoupled spectrum and shows two species: a broad signal at 188.5 ppm derived from EPSPS-bound glyphosate and a much narrower signal at 184.5 ppm from free glyphosate. The C-3 [^{13}C (99%), ^{15}N (99%)] glyphosate signal for the free species was a doublet of doublets at 58.58 ppm with J_{C-P} = 154.9 Hz and J_{C-N} = 5.1 Hz. The C-3 signal

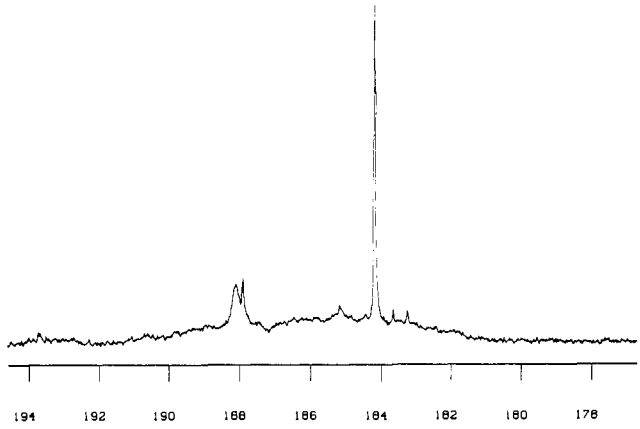


FIGURE 8: ^{13}C NMR of the dead-end complex (E^{S3P}_{Glyph}). The sample contained 1.6 mM EPSPS, 25 mM HEPES, 25 mM KCl, 2% glycerol, 2 mM β-mercaptoethanol, 0.01 mM EDTA, 2 mM ^{13}C (99%) C-1 glyphosate, and 10 mM S3P at pH 7.0 in 10% D_2O. The spectrum was acquired at 25 °C on a Varian XL 400 for 33 400 transients. The sharp signal present at 184.5 ppm is the C-1 signal for free glyphosate. The broadened signal at 188.5 ppm is the C-1 signal for glyphosate in the dead-end complex. The sharp signal just upfield at 188 ppm is the natural abundance signal for the carboxyl group of free S3P.

for the enzyme-bound form was not detectable.

The downfield δ perturbation for the carboxyl group as discussed above coincides with the electric field shift effect induced by the deprotonation of an α-ammonium ion. The EPSPS-induced deshielding perturbation for the ^{13}C NMR δ for glyphospate bound in the dead-end complex intersects the titration curve in Figure 6 at pH 10.1. Hence, for the carboxyl moiety, since a linear electric field shift cannot be invoked to explain the deshielding observed (a contradiction of the ^{15}N NMR data), then there is the possibility that the chemical shift change is related to a conformational effect induced by the enzyme.

Conclusions

The ^{13}C, ^{15}N, and ^{31}P NMR chemical shifts of glyphosate are reported for the entire pH range. The ^{31}P and ^{13}C NMR δ vs pH titration curves are interpreted by proposing two types of average solution structures for glyphosate as formulated on the basis of calculations: (1) an energy-minimized spirocyclic rotationally restricted conformation resulting from the formation of intramolecular salt bridges as intramolecular H-bonds between the oxygen anions of the phosphonate and carboxyl groups and the ammonium ion protons present in the pH range 0.0–9.0; (2) a linear structure that exists when glyphosate has a net charge of +1 below pH 0.0 or a net charge of −3 above pH 11.5. The $\Delta\delta$ observed in the ^{13}C, ^{15}N, and ^{31}P NMR titration curves are correlated to bond angles in the glyphosate backbone. The ^{31}P, ^{15}N, and ^{13}C δ values move downfield for increasing C–P–O, C–N–C, and C–C–O bond angles, respectively. We postulate that the chemical shift perturbations observed upon binding the enzyme can be explained by changes in the bond angles about the phosphonate, amine, and carboxyl moieties induced by EPSPS on the glyphosate backbone.

The ^{31}P NMR signal for glyphosate is compared to a series of model glyphosates, deazaglyphosate, (aminomethyl)-phosphonate, *N*-methylglyphosate, and *N*,*N*-dimethylglyphosate throughout the pH range. The customary shielding is observed for ionization of the phosphonate oxygens; however, a large deshielding is observed upon deprotonation of the ammonium ion. These two main features in the titration profile are correlated to the C–P–O bond angle by using calculations that resulted in the classification of glyphosate in two types of solution structures mentioned above. The ^{15}N NMR δ vs pH titration curve is also explained in terms of intramolecular H-bonding and proposed to correlate with the C–N–C bond angle also consistent with reported literature ^{15}N δ values for cyclic amines.

^{13}C, ^{15}N, and ^{31}P NMR have been used to characterize the herbicidal dead-end ternary complex of EPSPS, S3P, and glyphosate. The ^{13}C NMR data for glyphosate in the dead-end complex are not consistent with protonation of the carboxyl group upon binding. Protonation of the carboxyl group results in shielding, whereas a 5 ppm deshielding perturbation was measured. In addition, the deshielding observed for the glyphosate signal in the complex cannot be explained by the electric field effect shift induced upon deprotonation of the ammonium ion as observed for other α-amino acids as this is contrary to the ^{15}N NMR result. The deshielding for the ^{13}C carboxyl signal is also correlated to bond angle on the basis of calculations for the C–C–O bond. The J_{C_3-P} pH dependence reflects the ionization of both the phosphonate and ammonium groups whose ions are the primary determinant in the predicted structures by calculations. In addition, the $^3J_{C-P}$ pH dependence reflects specifically the ammonium protonation state, but does not show a measurable change for the carboxyl group ionization. The ^{13}C NMR data do suggest that conformations are changed upon ionization as measured by these J_{C-P} coupling constants. Hence, the ^{13}C NMR data support the proposal that the protein-induced chemical shift perturbations are linked to the bound conformation of glyphosate rather than a noncharacterizable environmental effect.

The ^{31}P and ^{15}N NMR data, supported by the calculations, predict that glyphosate is bound by EPSPS in the dead-end ternary complex as the full ammonium ion, but is not in the spirocyclic structure where the internal H-bonds are maintained. Rather, glyphosate is bound in a conformation equivalent to the average bond angles present free in solution at pH 10.1 or −0.8. These data do not distinguish if glyphosate is bound in a conformation similar to that required for PEP binding to the EPSPS–S3P binary complex as expected for a transition-state analogue. Likewise, other modes of glyphosate inhibition are not suggested.

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References

Amrhein, N., Deus, B., Gehrke, P., & Steinrucken, H. C. (1980) *Plant Physiol. 66*, 830–834.

Anderson, K. S., Sikorski, J. A., & Johnson, K. A. (1988a) *Biochemistry 27*, 1604–1610.

Anderson, K. S., Sikorski, J. A., & Johnson, K. A. (1988b) *Biochemistry 27*, 7395–7406.

Anton, D. L., Hedstrom, L., Fish, S. M., & Abeles, R. H. (1983) *Biochemistry 22*, 5903–5908.

Appleton, T. G., Hall, J. R., & McMahon, I. J. (1986) *Inorg. Chem. 25*, 726–734.

Asano, Y., Lee, J. J., Shieh, T. L., Kowal, C., & Floss, H. G. (1985) *J. Am. Chem. Soc. 107*, 4314–4320.

Baraniak, J., & Frey, P. A. (1988) *J. Am. Chem. Soc. 110*, 4059–4060.

Batchelor, J. G. (1975) *J. Am. Chem. Soc. 97*, 3410–3415.
Batchelor, J. G., Feeny, J., & Roberts, G. C. K. (1975) *J. Magn. Reson. 20*, 19–38.
Blomberg, F., Maurer, W., & Ruterjans, H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1409–1413.
Bondinell, W. E., Vnek, J., Knowles, P. E., Sprecher, M., & Sprinson, B. (1971) *J. Biol. Chem. 246*, 6191–6196.
Borthwick, P. (1980) *Acta Crystallogr. B36*, 628–632.
Clementi, E. (1967) *J. Chem. Phys. 47*, 2323–2333.
Cooper, R. A., Lichter, R. L., & Roberts, J. D. (1973) *J. Am. Chem. Soc. 95*, 3724–3729.
Cornelius, R. D., Plant, P. A., & Cleland, W. W. (1977) *Inorg. Chem. 16*, 2799–2805.
Cornish-Bowden, A. (1986) *FEBS Lett. 203*, 3–6.
della-Cioppa, G. R., & Kishore, G. M. (1988) *EMBO J. 7*, 1299–1306.
della-Cioppa, G. R., Bauer, S. C., Klein, B. K., Shah, D. M., Fraley, R. T., & Kishore, G. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6873–6877.
Duthaler, R. O., & Roberts, J. D. (1978) *J. Am. Chem. Soc. 100*, 3882–3889, 3889–3895.
Feeney, J., Batchelor, J. G., Albrand, J. P., & Roberts, G. C. K. (1979) *J. Magn. Reson. 33*, 519–529.
Fersht, A. (1987) *Trends Biochem. Sci. 12*, 301–304.
Franz, J. E. (1985) *The Herbicide Glyphosate* (Grossbard, E., & Atkinson, D., Eds.) pp 1–17, Butterworth, Boston, MA.
Gorenstein, D. G. (1984) *Phosphorus-31 NMR, Principles and Applications* (Gorenstein, D. G., Ed.) pp 1–33, Academic Press, Orlando, FL.
Grimshaw, C. E., Sogo, S. G., & Knowles, J. R. (1982) *J. Biol. Chem. 257*, 596–598.
Grimshaw, C. E., Sogo, S. G., Copley, S. D., & Knowles, J. R. (1984) *J. Am. Chem. Soc. 106*, 2699–2700.
Gutfreund, H. (1972) *Enzymes: Physical Principles*, Chapter 2, Wiley, London.
Heubel, P.-H. C., & Popov, A. I. (1979) *J. Solution Chem. 8*, 615–625.
Horsley, W., & Sternlicht, H. (1968) *J. Am. Chem. Soc. 90*, 3738–3748.
Horsley, W., Sternlicht, H., & Cohen, J. S. (1970) *J. Am. Chem. Soc. 92*, 680–686.
Huynh, Q. K. (1987) *Arch. Biochem. Biophys. 258*, 233–239.
Huynh, Q. K., Kishore, G. M., & Bild, G. S. (1988) *J. Biol. Chem. 263*, 735–739.
Kahan, F. M., Kahan, J. S., Cassidy, P. J., & Kropp, H. (1974) *Ann. N.Y. Acad. Sci. 235*, 364–385.
Kishore, G. M., & Shah, D. M. (1988) *Annu. Rev. Biochem. 57*, 627–663.
Kishore, G. M., Brundage, L., Kolk, K., Padgette, S. R., Rochester, D., et al. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1506.
Knuuttila, P., & Knuuttila, H. (1979) *Acta Chem. Scand. B33*, 623–626.
Kricheldorf, H. R. (1979) *Org. Magn. Reson. 12*, 414–417.
Kurz, L. C., & Frieden, C. (1987) *Biochemistry 26*, 8450–8457.
Lanzetta, P. A., Alvarez, L. J., Reinah, P. S., & Dandia, O. A. (1979) *Anal. Biochem. 100*, 95–97.
Lee, J. J., Asano, Y., Spreafico, F., Lee, K., & Floss, H. G. (1984) *J. Am. Chem. Soc. 106*, 3367–3368.
Leipert, T. K., & Noggle, J. H. (1975) *J. Am. Chem. Soc. 97*, 269–272.
Levin, J. G., & Sprinson, B. (1964) *J. Biol. Chem. 239*, 1142–1150.
Levy, G. C., & Lichter, R. L. (1979) *Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy* (Levy, G. C., & Lichter, R. L., Eds.) pp 37–52, Wiley, New York.
Lewendon, A., & Coggins, J. R. (1983) *Biochem. J. 213*, 187–191.
Lichter, R. L., & Roberts, J. D. (1972) *J. Am. Chem. Soc. 94*, 2495–2500.
London, R. E. (1980) *J. Magn. Reson. 38*, 173–177.
London, R. E., Walker, T. E., Kollman, V. H., & Maywiyoff, N. A. (1978) *J. Am. Chem. Soc. 100*, 3723–3729.
MacDonald, J. C., Bishop, G. G., & Mazurek, M. (1976) *Can. J. Chem. 54*, 1226–1233.
Madsen, H. E. L., Christensen, H. H., & Gottlieb-Petersen, C. (1978) *Acta Chem. Scand. A32*, 79–83.
Motekaitis, R. J., & Martell, A. E. (1985) *J. Coord. Chem. 14*, 139–149.
Mousdale, D. M., & Coggins, J. R. (1984) *Planta 169*, 78–83.
Padgette, S. R., Huynh, Q. K., Borgmeyer, J., Shah, D. M., Brand, L. A., Re, D. B., Bishop, B. F., Rogers, S. G., Fraley, R. T., & Kishore, G. M. (1987) *Arch. Biochem. Biophys. 258*, 564–573.
Padgette, S. R., Huynh, Q. K., Aykent, S., Sammons, R. D., Sikorski, J. A., & Kishore, G. M. (1988a) *J. Biol. Chem. 263*, 1798–1802.
Padgette, S. R., Smith, C. E., Huynh, Q. K., & Kishore, G. M. (1988b) *Arch. Biochem. Biophys. 266*, 254–262.
Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527.
Pittard, J., & Ely, B. (1979) *J. Bacteriol. 138*, 933.
Rabenstein, D. L., & Sayter, T. L. (1976) *J. Magn. Reson. 24*, 27.
Ratcliffe, C. I., Ripmeester, J. A., & Tse, J. S. (1983) *Chem. Phys. Lett. 99*, 177–180.
Rogers, S. G., Brand, L. A., Holder, S. B., Sharps, E. S., & Bracken, M. J. (1983) *Appl. Environ. Microbiol. 1*, 37–43.
Rogers, T. E. (1986) U.S. Patent 4 568 432.
Rueppel, M. L., & Marvel, J. T. (1976) *Org. Magn. Reson. 8*, 19–20.
Sapozhnikova, N. A., Strunin, B. P., Maslennikov, E. I., Yasman, Y. B., & Siminov, V. D. (1986) (USSR) *Zh. Obshch. Khim. 56*, 2277–2280.
Sheldrick, V. W. S., & Morr, M. (1981) *Acta Crystallogr. B37*, 733–734.
Shkolnikova, L. M., Porai-Koshits, M. A., Dyatlova, N. M., Yaroshenko, G. F., & Rudomino, M. V. (1982) *Zh. Strukt. Khim. 23*, 98–107.
Shoval, S., & Yariv, S. (1981) *Agrochimica 25*, 377–386.
Smith, P. H., Hahn, F. E., Hugi, A., & Raymond, K. N. (1989) *Inorg. Chem.* (in press).
Stalker, D. M., Hiatt, W. R., & Comai, L. (1985) *J. Biol. Chem. 260*, 4724–4728.
Steinrucken, H. C., & Amrhein, N. (1980) *Biochem. Biophys. Res. Commun. 94*, 1207–1212.
Steinrucken, H. C., & Amrhein, N. (1984) *Eur. J. Biochem. 143*, 341–349, 351–357.
Street, I. P., Armstrong, C. R., & Withers, S. G. (1986) *Biochemistry 25*, 6021–6027.
Suprenant, H. L., Sarneski, J. E., Key, R. E., Byrd, J. T., & Reilley, C. N. (1980) *J. Magn. Reson. 40*, 231–243.
Warburg, O., & Christian, W. (1941–1942) *Biochem. Z. 310*, 384.
Wasielewski, C., & Antezak, K. (1981) *Synthesis 7*, 540–541.
Webb, G. A. (1986) *Annu. Rep. NMR Spectrosc. 18*, 246.
Westheimer, F. H., & Schmidt, D. E., Jr. (1971) *Biochemistry 10*, 1249–1253.

Wibbenmeyer, J., Brundage, L., Padgette, S. R., Likos, J. J., & Kishore, G. M. (1988) *Biochem. Biophys. Res. Commun. 153*, 760–766.
Witanowski, M., Stefaniak, L., & Januszawski, H. (1973) *Nitrogen Chemical Shifts in Organic Compounds, in Nitrogen NMR* (Witanowski, M., & Webb, G. A., Eds.) pp 163–260, Plenum Press, New York.
Zamenhoff, S. (1957) *Methods Enzymol. 3*, 696.
Zemell, R. I., & Anwar, R. A. (1975) *J. Biol. Chem. 250*, 4959–4964.
Zyablikova, T. A., Magdeer, I. M., & Shermergorn, I. M. (1969) *Izv. Akad. Nauk SSSR, Ser. Khim. 3*, 694–696.

Recognition of $\beta\beta'$-Substituted and $\alpha\beta,\alpha'\beta'$-Disubstituted Phosphonate Analogues of Bis(5′-adenosyl) Tetraphosphate by the Bis(5′-nucleosidyl)-tetraphosphate Pyrophosphohydrolases from *Artemia* Embryos and *Escherichia coli*†

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ABSTRACT: A total of 13 phosphonate analogues of bis(5′-adenosyl) tetraphosphate (AppppA) have been tested as substrates and inhibitors of the asymmetrically cleaving bis(5′-nucleosidyl) tetraphosphatase (NppppNase) from *Artemia* and the symmetrically cleaving NppppNase from *Escherichia coli*. With the *Artemia* enzyme, the substrate efficiency of $\beta\beta'$-substituted compounds decreased with decreasing substituent electronegativity (O > CF_2 > CHF > CCl_2 > CHCl > CH_2) such that AppCF_2ppA and AppCH_2ppA were hydrolyzed at 70% and 2.5% of the rate of AppppA, respectively. These compounds were competitive inhibitors of this enzyme with K_i values that generally also decreased with electronegativity from 12 μM for AppCF_2ppA to 0.4 μM for AppCH_2ppA (K_m for AppppA = 33 μM). AppCH=CHppA and AppCH_2CH_2ppA were neither effective substrates nor inhibitors of the *Artemia* enzyme. $\alpha\beta,\alpha'\beta'$-Disubstituted analogues were generally less effective inhibitors with K_i values ranging from 23 μM (AppCH_2ppCH_2pA) to > 1.5 mM (AppCH_2CH_2ppCH_2CH_2pA). However, they displayed a low and unexpected rate of *symmetrical* cleavage by the *Artemia* enzyme: e.g., ApCHFppCHFpA yielded ApCHFp at 3% of the rate of AppppA breakdown. Both sets of analogues were also competitive inhibitors of the *E. coli* NppppNase with K_i values ranging from 7 μM (AppCH_2ppA) to 250 μM (AppCH_2CH_2ppCH_2CH_2pA) (K_m for AppppA = 28 μM). The only $\alpha\beta,\alpha'\beta'$-disubstituted analogue to be hydrolyzed by the *E. coli* enzyme was ApCF_2ppCF_2pA at 0.2% of the rate of AppppA; however, several of the $\beta\beta'$-substituted compounds showed a limited degree of *asymmetrical* cleavage. These results are interpreted in terms of a "frameshift" model for substrate binding in which the oligophosphate moiety of the substrate can position itself in the active site of either enzyme with either P^α or P^β adjacent to the attacking nucleophile depending on the electronegativity of the phosphonate substituent.

In 1963, the first bis(5′-nucleosidyl) oligophosphate, bis(5′-guanosyl) tetraphosphate (GppppG),[1] was isolated from encysted embryos of the brine shrimp *Artemia*, where it serves as a purine ring store for the developing organism (Finamore & Warner, 1963; Warner, 1980). Since then, several other nucleotides of general structure Np_nN' have been detected in a wide variety of prokaryotes and eukaryotes, but their functions remain to be clearly established. Interest has focused mainly on the adenine-containing members of this family and in particular bis(5′-adenosyl) tetraphosphate (AppppA). This nucleotide was originally implicated in the initiation of DNA replication [reviewed in Zamecnik (1983), McLennan and Prescott (1984), Baril et al. (1985), and Grummt (1988)]; however, evidence to the contrary also exists (Bambara et al., 1985; Plateau et al., 1987), and this has led to the search for alternative functions.

For example, AppppA and a number of other adenylated nucleotides including ApppA, AppppG, ApppGpp, and ApppG accumulate rapidly in bacteria when they are subjected to metabolic stresses such as heat shock, oxidation, and organic solvents (Lee et al., 1983a,b; Bochner et al., 1984; VanBogelen et al., 1987; Balodimos et al., 1988); however, with the possible exception of the *oxy* R-controlled oxidation stress regulon of

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[1] Abbreviations: GppppG, bis(5′-guanosyl) tetraphosphate; AppppA, bis(5′-adenosyl) tetraphosphate; other bis(5′-nucleosidyl) oligophosphates are abbreviated in a similar fashion; NppppNase, bis(5′-nucleosidyl)-tetraphosphate pyrophosphohydrolase; AppCH_2ppA, diadenosine 5′,5‴-P^1,P^4-(P^2,P^3-methylene)tetraphosphate; AppCH_2p, adenosine 5′-(β,γ-methylene)triphosphate; AppCH_2ppCH_2pA, diadenosine 5′,5‴-P^1,P^4-(P^1,P^2-methylene-P^3,P^4-methylene)tetraphosphate; AppCH_2p, adenosine 5′-(α,β-methylene)diphosphate; other phosphonate analogues of AppppA, ATP, and ADP are abbreviated in a similar fashion.

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