Evaluation of 5-Enolpyruvoylshikimate-3-phosphate Synthase Substrate and Inhibitor Binding by Stopped-Flow and Equilibrium Fluorescence Measurements†

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Appendix: Derivation of the Apparent Dissociation Constant for Shikimate 3-Phosphate Binding in the Presence of Glyphosate

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ABSTRACT: The binding of substrates and the herbicide *N*-(phosphonomethyl)glycine (glyphosate) to enolpyruvoylshikimate-3-phosphate (EPSP) synthase was evaluated by stopped-flow and equilibrium fluorescence measurements. Changes in protein fluorescence were observed upon the binding of EPSP and upon the formation of the enzyme–shikimate 3-phosphate–glyphosate ternary complex; no change was seen with either shikimate 3-phosphate (S3P) or glyphosate alone. By fluorescence titrations, the dissociation constants were determined for the formation of the enzyme binary complexes with S3P ($K_{d,S}$ = 7 ± 1.2 μM) and EPSP ($K_{d,EPSP}$ = 1 ± 0.01 μM). The dissociation constant for S3P was determined by competition with EPSP or by measurements in the presence of a low glyphosate concentration. At saturating concentrations of S3P, glyphosate bound to the enzyme–S3P binary complex with a dissociation constant of 0.16 ± 0.02 μM. Glyphosate did not bind significantly to free enzyme, so the binding is ordered with S3P binding first:

$$E \underset{k_{-1}}{\overset{k_1[S]}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2[G]}{\rightleftharpoons}} E{\cdot}S{\cdot}G^*$$

where S refers to S3P, G refers to glyphosate, and E·S·G* represents the complex with altered fluorescence. The kinetics of binding were measured by stopped-flow fluorescence methods. The rate of glyphosate binding to the enzyme–S3P complex was $k_2 = (7.8 \pm 0.2) \times 10^5$ M^{-1} s^{-1}, from which we calculated the dissociation rate $k_{-2} = 0.12 \pm 0.02$ s^{-1}. The rate of S3P binding was estimated by fluorescence measurements in the presence of glyphosate; the data indicated a rapid equilibrium binding of S3P with the lower limits of $k_1 \geq 2 \times 10^8$ M^{-1} s^{-1} and $k_{-1} \geq 1400$ s^{-1}. The rates of binding and dissociation for EPSP were too fast to measure and suggest a rapid equilibrium binding with values greater than 6×10^7 M^{-1} s^{-1} and 60 s^{-1}, respectively. The information gained from this study provides thermodynamic and kinetic data needed to evaluate the utilization of substrate binding energy in catalysis and in the interaction of glyphosate in the ternary complex.

Glyphosate,[1] the active ingredient of the herbicide Roundup, has been shown to elicit its herbicidal action by the inhibition of EPSP synthase (Steinrucken & Amrhein, 1980), an enzyme in the pathway for biosynthesis of aromatic amino acids in plants and bacteria. In order to better understand the catalytic mechanism of EPSP synthase and the interaction of glyphosate with the enzyme, a basic understanding of substrate and inhibitor binding is required.

EPSP synthase catalyzes the transfer of the enolpyruvoyl moiety from phosphoenolpyruvate (PEP) to the hydroxyl group of carbon 5 of shikimate 3-phosphate (S3P) with the elimination of phosphate to produce 5-enolpyruvoylshikimate 3-phosphate (EPSP):

$$S3P + PEP \rightleftharpoons EPSP + P_i$$

The steady-state kinetics of *Neurospora* EPSP synthase have been examined by Boocock and Coggins (1983). The kinetic patterns suggest an ordered, sequential mechanism in which S3P precedes the binding of PEP. In addition, the inhibition kinetics with glyphosate suggested a reversible interaction between glyphosate and the enzyme–S3P complex, in a reaction that is competitive versus PEP and uncompetitive versus S3P.

It has been suggested that glyphosate is a transition-state analogue for PEP in the reaction with S3P (Cromartie, 1986; Steinrucken & Amrhein, 1980). However, there is no quantitative data to allow a comparison of the rates or free energies of binding of glyphosate with those parameters for PEP. In the absence of such information, one cannot hope to distinguish

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[1] Abbreviations: dideoxy-S3P, 4,5-dideoxyshikimic acid 3-phosphate; EPSP, 5-enolpyruvoylshikimate 3-phosphate; glyphosate, *N*-(phosphonomethyl)glycine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; S3P, shikimate 3-phosphate; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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whether glyphosate should be considered as a ground-state or a transition-state analogue. This distinction is important in order to understand the high degree of specificity that glyphosate has for EPSP synthase over other PEP-utilizing enzymes and to establish the mechanism of this unusual reaction. Few mechanistic studies have been performed, and the results have left open the question as to whether the reaction proceeds as a direct addition–elimination mechanism with a tetrahedral intermediate (Bondinell et al., 1971) or whether there is a reactive covalent intermediate formed from PEP (Anton et al., 1983). Only one other enzyme is known to transfer an enolpyruvoyl moiety from PEP; pyruvate–UDP-*N*-acetylglucosamine transferase catalyzes the first commited step in the biosynthesis of bacterial cell wall peptidoglycan in a reaction that proceeds via a covalent enzyme–enolpyruvate intermediate (Zemell, 1975) and is not inhibited by glyphosate.

In order to understand the nature of the interaction of glyphosate with EPSP synthase, more quantitative data on the free energy of binding of S3P, glyphosate, and EPSP are required. With the substrate and inhibitor binding energies in hand, the net contributions of individual binding interactions of key contact points can be estimated. In addition, the manner in which EPSP synthase utilizes the binding energy to carry out chemical catalysis can be better understood.

In this report, stopped-flow and equilibrium fluorescence methods were used to estimate the rates of binding and dissociation constants for S3P, EPSP, and glyphosate.

Materials and Methods

Enzyme Purification. EPSP synthase was isolated from a cloned *Escherichia coli* strain that overproduces the enzyme. The purification procedure employed is outlined as follows: (1) protamine sulfate precipitation to remove nucleic acid as described by Pittard et al. (1979); (2) ammonium sulfate fractionation (50–70%); (3) phenyl-Sepharose CL-4B chromatography with elution using a linear gradient of 0.8–0.0 M ammonium sulfate in 25 mM Tris-HCl, pH 7.5; and (4) Mono-Q chromatography (HR 10/10 column, Pharmacia) using a 30-min linear gradient of 0–300 mM KCl in 25 mM Tris-HCl, pH 7.5. The specific activity of the final protein solution after concentration was 70 μmol/(min·mg) (53 s^{-1}) as determined by assaying the release of inorganic phosphate by the method of Lanzetta et al. (1979). The enzyme concentration was determined by using an extinction coefficient of 0.77 cm^2/mg at 280 nm. A molecular weight of 45 000 was used for calculation of enzyme active site concentration.

Chemicals. Shikimate 3-phosphate (S3P) was synthesized enzymatically by treatment of shikimic acid (Sigma Chemical Co.) with shikimate kinase. S3P was purified by chromatography on DEAE-Sephadex A-25 eluted with a gradient of triethylammonium bicarbonate. The sodium salt of S3P was obtained by passing through a Dowex-50 column (Na^+ form). The S3P was standardized by NMR with 1-methyltriazole as an internal standard.

In the NMR standardization method, preweighed samples of 1-methyl-1*H*-1,2,4-triazole and S3P were diluted with D_2O, so the concentration of methyltriazole was known. A spectrum of this mixture was then taken, and the area for the proton in the 5-position of the triazole appearing at 8.0 ppm was compared with the area for the vinyl shikimate ring proton for S3P which appeared at 7.2 ppm.

5-Enolpyruvoylshikimate 3-phosphate (EPSP) was synthesized enzymatically from S3P and phosphoenolpyruvate PEP) (obtained from Sigma Chemical Co.) by using EPSP synthase. Purification and standardization was accomplished in a manner similar to that described for S3P.

Glyphosate was obtained from Monsanto internal stocks and was analytically pure (>99%).

All buffers and other reagents employed were of highest commercial purity. Millipore ultrapure water was used for all solutions. All experiments were conducted at 20 °C in a buffer containing 50 mM HEPES and 50 mM KCl at pH 7.0.

Fluorescence Titration Experiments. Equilibrium fluorescence measurements were made using an SLM 8000 fluorometer (Urbana, IL) at 20 °C. The excitation wavelength was 280 nm (1-nm band-pass), and the emission wavelength was 360 nm (8-nm band-pass). The decrease in fluorescence at 360 nm, due to ligand binding, was monitored. In a typical titration experiment, 0.5-μL aliquots of ligand were added to a 2.0-mL enzyme solution. A protein concentration of 0.5 μM was chosen for all the titration experiments in order to maintain a low protein concentration relative to the K_d value; this is a requirement for accurate determination of the dissociation constant (Edsall & Gutfreund, 1983; Norby et al., 1980). The solution was mixed after the addition of each aliquot, and the fluorescence intensity was recorded as the average of four 10-s readings. Ligand was added until no further decrease in fluorescence was observed; in each case the data extend to higher ligand concentrations than are shown in the figures. Each titration experiment was repeated 2–3 times. The observed fluorescence (corrected for dilution) was then plotted versus total ligand concentration. The data were then fit to the quadratic equation by nonlinear regression using the following expression relating observed fluorescence to substrate concentration $[S_0]$:

$$F = F_0 + (\Delta F/E_0)\{(K_d^{app} + [E_0] + [S_0]) - \sqrt{(K_d^{app} + [E_0] + [S_0])^2 - 4[E_0][S_0]}\}/2 \quad (1)$$

The total enzyme concentration E_0 was constant (0.5 μM). The dissociation constant (K_d), initial fluorescence (F_0), and the total change in fluorescence (ΔF) were obtained as the least-squares fit of each data set to the above equation by nonlinear regression. In each figure shown, the fluorescence intensity was normalized by dividing by the fitted parameter, F_0.

Stopped-Flow Experiments. Stopped-flow measurements were performed by using an apparatus designed and built at The Pennsylvania State University as described by Johnson (1986). This apparatus has a 1.6-ms dead time, a 2-mm path length, and a thermostated observation cell maintained at 20 °C. Enzyme–substrate complex formation was monitored by following protein fluorescence with a 280-nm interference filter on the input and monitoring the quenching of intrinsic enzyme fluorescence with an output filter at 360 nm. In most experiments an average of four runs was used for data analysis and a minimum of a 5-fold excess of the variable substrate over enzyme was used to allow analysis as a pseudo-first-order rate constant. The data were collected over a given time interval by a computer. Rate constants were obtained by fitting the data to a single exponential by an iterative, nonlinear least-squares program using a modification of the method of moments (Johnson, 1986).

Measurement of S3P Binding Rates. The rate of S3P binding to EPSP synthase was determined in the presence of a fixed concentration of glyphosate. In these experiments an enzyme solution (0.5–6 μM) containing 500 μM glyphosate was mixed with an equal volume of S3P solution (2.5–100 μM). The rate of formation of the ternary enzyme–S3P–glyphosate complex was then obtained at varying concentrations of S3P. As described under Results (see Scheme II), the data indicated that the binding of S3P occurred as a rapid

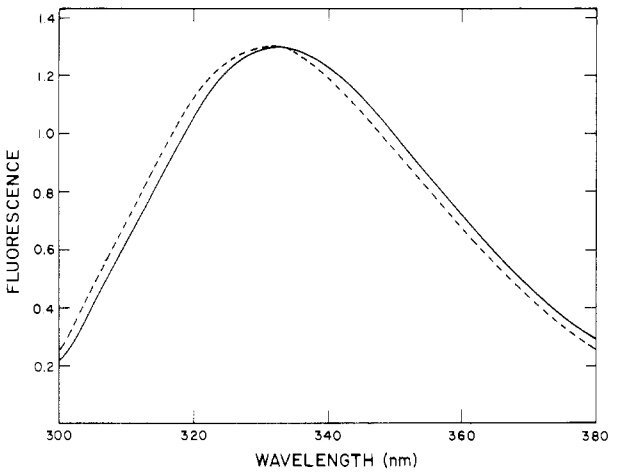

FIGURE 1: Fluorescence emission spectra of EPSP synthase with EPSP. Fluorescence emission spectra are shown for 0.5 μM enzyme (—) and enzyme with 50 μM EPSP (---). The relative fluorescence intensity is shown in arbitrary units.

equilibrium step, followed by glyphosate binding to produce a change in fluorescence. Therefore, the concentration dependence of the rate of binding was fit to a hyperbola, $k_{obsd} = k_2[G][S]/(K_{d,S} + [S])$, where $K_{d,S}$ is the dissociation constant for S3P and k_2 is the binding rate for glyphosate.

In one experiment to measure the rate of S3P binding at high glyphosate concentration, only a 2-fold excess of S3P over enzyme was used in order to obtain a measurable rate. The data were then fit to the complete kinetic pathway (Scheme II) by computer simulation using the program KINSIM (Barshop et al., 1983). In the trial and error fitting process, the values of k_2 and k_{-2} were held constant (0.78 μM^{-1} s^{-1} and 0.12 s^{-1}, respectively) and the values of k_1 and k_{-1} were adjusted to fit the data while keeping the ratio $k_{-1}/k_1 = K_{d,S} = 7$ μM.

Measurement of the Rate of Glyphosate Binding to Enzyme–S3P. The rate of glyphosate binding to the enzyme–S3P complex was determined by mixing an enzyme solution (4.5–9 μM) containing 500 μM S3P with an equal volume of glyphosate solution (20–500 μM). The rate of binding was then determined at varying concentrations of glyphosate. The observed rate was plotted versus the final concentration of glyphosate (after mixing) and fit to the equation $k_{obsd} = k_2[G] + k_{-2}$, where k_2 is the rate of glyphosate binding, [G] is the concentration of glyphosate, and k_{-2} is the dissociation rate according to Scheme II (see Results).

RESULTS

Equilibrium Binding of EPSP. The equilibrium constant for EPSP binding to EPSP synthase can be determined directly because there is a small decrease in fluorescence and a blue shift in the maximum wavelength of emission upon addition of EPSP to the enzyme solution (Parr et al., 1987) (Figure 1). The fluorescence change at 360 nm was titrated to determine the dissociation constant as shown in Figure 2. The data were fit to the quadratic equation by nonlinear regression to obtain the dissociation constant of 1.02 ± 0.01 μM for EPSP.

Equilibrium Binding of S3P and Glyphosate. Changes in fluorescence emission after addition of S3P or S3P plus glyphosate are shown in Figure 3. There is no change in intrinsic protein fluorescence upon addition of S3P at any concentration examined (2 mM highest), and no change is seen with glyphosate alone (data not shown). There is, however, a decrease in observed fluorescence and a blue shift upon the addition of glyphosate in the presence of S3P. Because glyphosate does not bind detectably in the absence of S3P (unpublished data), the binding must be ordered with S3P binding first as shown in Scheme I. The dissociation constants for S3P ($K_{d,S}$) and glyphosate ($K_{d,G}$) can be determined by titrations under two

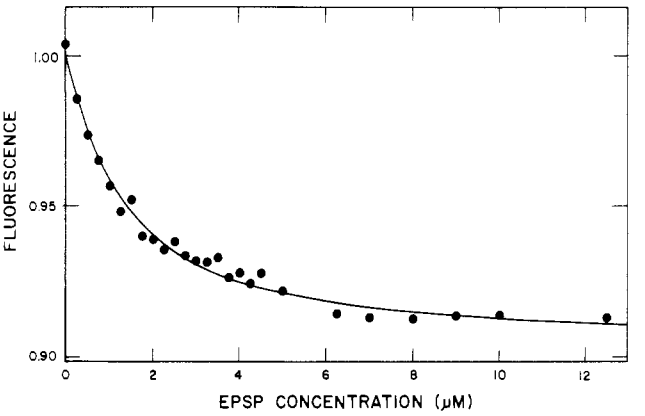

FIGURE 2: Fluorescence titration of EPSP binding. The decrease in fluorescence was monitored as EPSP was added in small increments to a solution of enzyme (0.5 μM) until no further change was noted. The smooth line shows the fit to a quadratic equation as described under Materials and Methods with a $K_d = 1.02 \pm 0.01$ μM. The total change represented a 9.6% decrease.

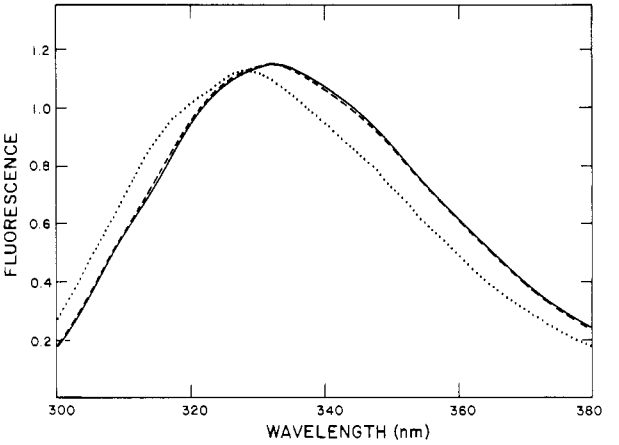

FIGURE 3: Fluorescence emission spectra of EPSP synthase with S3P and glyphosate. Fluorescence emission spectra are shown for 0.5 μM enzyme (—), enzyme with 50 μM S3P (---), and enzyme with 50 μM S3P and 50 μM glyphosate (···). The relative fluorescence intensity is shown in arbitrary units.

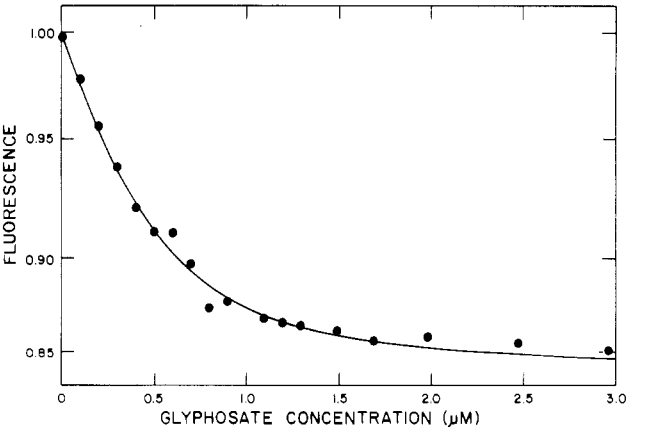

FIGURE 4: Fluorescence titration of glyphosate binding. The dissociation constant for glyphosate binding to the enzyme–S3P binary complex was determined by titrating glyphosate into a solution of 0.5 μM enzyme containing 250 μM S3P. The data were fit to the quadratic equation as described under Materials and Methods, giving a $K_{d,G} = 0.16 \pm 0.02$ μM. The total change represented a 16% decrease.

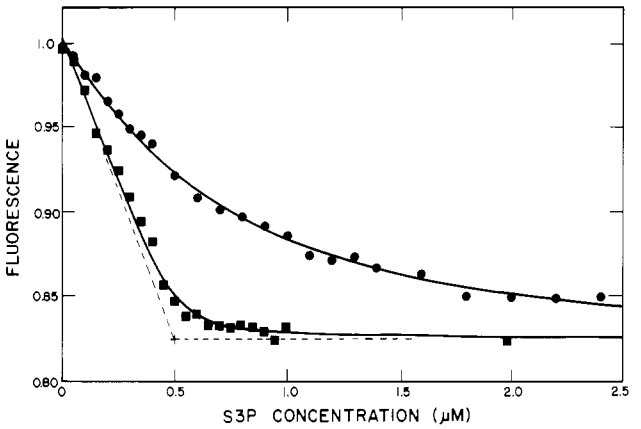

FIGURE 5: Apparent binding constant for S3P. Two titration curves are shown performed by the addition of S3P to an enzyme solution (0.5 μM) containing 2.5 (●) or 100 μM (■) glyphosate. The apparent dissociation constant varies leading to apparently tighter binding at higher glyphosate concentration as described in the text. The apparent K_d values obtained by quadratic fits were 0.405 ± 0.030 μM and 0.0116 ± 0.0043 μM, at 2.5 and 100 μM glyphosate, respectively.

limiting conditions. Titration of glyphosate into a solution of enzyme saturated with S3P provides a direct measurement of the dissociation constant for the binding of glyphosate to the enzyme–S3P binary complex ($K_{d,G}$). As shown in Figure 4, fluorescence titration of glyphosate into a solution of enzyme containing 250 μM S3P provides a measurement of $K_{d,G}$ = 0.16 ± 0.02 μM.

Scheme I

$$E + S \rightleftharpoons ES \qquad K_{d,S} = \frac{[E][S]}{[ES]}$$

$$ES + G \rightleftharpoons ESG \qquad K_{d,G} = \frac{[ES][G]}{[ESG]}$$

Titration of S3P into an enzyme solution containing an appropriate concentration of glyphosate (see below) as described in the Appendix provides an apparent dissociation constant defined by

$$K_{d,S}^{app} = K_{d,S}K_{d,G}/[G] \qquad (2)$$

where [G] represents glyphosate concentration. This expression shows that the titration of S3P into a solution of enzyme at a fixed glyphosate concentration will provide a value for $K_{d,S}^{app}$ that will vary with glyphosate concentration. According to this expression, we can solve for $K_{d,S}$ (binary complex of enzyme–S3P) from $K_{d,S}^{app}$, the concentration of glyphosate, and the value of $K_{d,G}$ measured independently.

In conducting these experiments, it was found that the concentration of glyphosate was important. Initially the titration was carried out in the presence of 100 μM glyphosate. This revealed a curve typical of very tight binding as shown in Figure 5. The titration was then conducted in the presence of 5 μM glyphosate (not shown) and 2.5 μM glyphosate (Figure 5). This comparison demonstrates the glyphosate concentration dependence of the apparent dissociation constant for S3P. The titration conducted in the presence of 2.5 μM glyphosate provided an apparent dissociation constant of 0.40 ± 0.03 μM. By use of the value of $K_{d,G}$ = 0.16 ± 0.02 μM and [G] = 2.5 μM, the value of $K_{d,S}$ was determined to be 6.3 ± 1.2 μM.

The binding of S3P at 100 μM glyphosate is too tight for accurate determination of the dissociation constant; error analysis during nonlinear regression indicated a 40% error in the K_d value from the fitting process alone. Because the accuracy of the K_d determination is a function of the deviation of the data from the limiting asymptotes for infinitely tight binding (Figure 5, dashed line), the dissociation constant calculated from this curve is extremely inaccurate. More importantly, under these conditions the value obtained for the dissociation constant is very sensitive to errors in the protein concentration. Variation of the enzyme concentration of ±10% produced 3- or 4-fold changes in the calculated K_d. In contrast, a similar change in the fitting of the data at 2.5 μM glyphosate produced only a 7% change in the calculated K_d. The titration at 100 μM glyphosate (or higher) does however provide an accurate estimation of the stoichiometry of one S3P per enzyme in the complex. Similarly, titrations at high enzyme concentration have established the stoichiometry involving one glyphosate per enzyme site.

Table I: Kinetic and Thermodynamic Constants[a]

ligand	K_d (μM)	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	$\Delta G°$ (kcal/mol)
S3P	7 ± 1.2	≥200	≥1400	−7.1
EPSP	1.0 ± 0.01	≥60	≥60	−8.0
glyphosate	0.16 ± 0.02	0.78 ± 0.02	0.12 ± 0.02	−9.1

[a] Kinetic and thermodynamic constants for EPSP synthase were determined in 50 mM HEPES and 50 mM KCl, pH 7.0, at 20 °C.

The value of $K_{d,S}$ was verified by a competition experiment between EPSP and S3P. For practical reasons due to a small amount of absorbance by high concentrations of S3P, it was best to do the competition by preincubating the enzyme with S3P and then titrating in EPSP. In this competition experiment, the apparent K_d value for EPSP was measured in the presence of a fixed concentration of S3P in excess over enzyme ($[S_0] \gg [E_0]$). Under these conditions, the apparent dissociation constant for EPSP is equal to

$$K_{d,EPSP}^{app} = K_{d,EPSP}(1 + [S_0]/K_{d,S}) \qquad (3)$$

where S represents S3P and $K_{d,EPSP}$ and $K_{d,S}$ are the true dissociation constants for EPSP and S3P, respectively. The actual value for $K_{d,EPSP}$ has been determined directly as described above. At 35 μM S3P, $K_{d,EPSP}^{app}$ was found to be 5.7 ± 0.6 μM (data not shown). Substituting these values back into eq 3 gives a $K_{d,S}$ of 7.6 ± 1.1 μM, which agrees very well with the $K_{d,S}$ = 6.3 ± 1.2 μM obtained in the previously described glyphosate experiment.

A summary of K_d values for the binary and ternary complexes appears in Table I. The calculated free energy of binding for the formation of each complex also appears in this table.

Rate of Glyphosate Binding to the Enzyme–S3P Complex. The binding of glyphosate to EPSP synthase is a two-step process that can be described as shown in Scheme II, where E·S·G* represents the ternary complex with altered fluorescence emission. If the enzyme is first saturated with S3P, the rate of glyphosate binding to the enzyme–S3P binary complex can be determined directly by stopped-flow fluorescence measurements. The reaction was initiated by mixing a preformed enzyme–S3P complex with various concentrations of glyphosate. A representative stopped-flow trace is shown in Figure 6. The data could be fit by a single exponential at each concentration examined.

Scheme II

$$E \underset{k_{-1}}{\overset{k_1[S]}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2[G]}{\rightleftharpoons}} E{\cdot}S{\cdot}G^*$$

The rate of glyphosate binding to the enzyme–S3P complex increased linearly with glyphosate concentration and showed

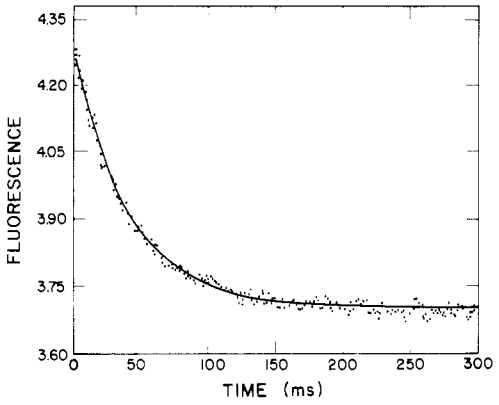

FIGURE 6: Kinetics of formation of the enzyme–S3P–glyphosate complex. The data are a representative trace from a stopped-flow measurement showing the time dependence of quenching of the intrinsic enzyme fluorescence after mixing 30 μM glyphosate with a preformed complex of 4.5 μM enzyme and 250 μM S3P (all concentrations are those after mixing). The smooth line shows the fit to a single exponential with a rate of 24.5 s^{-1}.

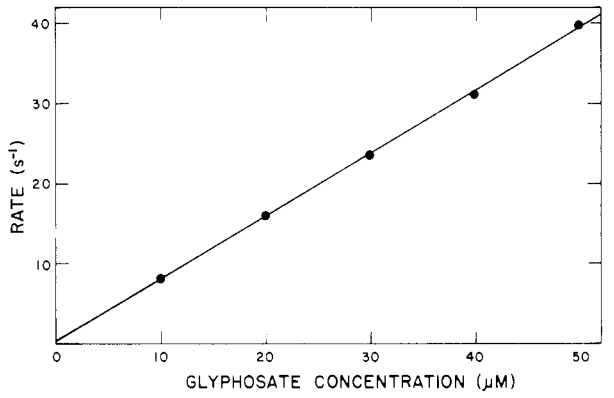

FIGURE 7: Rate of glyphosate binding to the enzyme–S3P complex. The rate of glyphosate binding to a preformed enzyme–S3P complex was measured by mixing various concentrations of glyphosate with enzyme presaturated with S3P (250 μM after mixing). To optimize the signal, the enzyme concentration was varied from 2.2 to 4.4 μM (concentration after mixing) in the experiments from the lower (<20 μM) to higher glyphosate concentrations. The observed rate of binding under pseudo-first-order conditions is approximated by $k_{obsd} = k_2[G] + k_{-2}$. The best fit to the data gives $k_2 = 0.78 \pm 0.02$ μM^{-1} s^{-1} and $k_{-2} = 0.44 \pm 0.77$ s^{-1}. Because of large errors in calculation of the intercept, the best estimate of the dissociation rate is $k_{-2} = 0.12$ s^{-1}, calculated from the $K_{d,G} = 0.16$ μM.

no sign of approaching a maximum rate (Figure 7). The observed rate of association under pseudo-first-order conditions and with saturating S3P may be approximated by $k_{obsd} = k_2[G] + k_{-2}$, where k_2 and k_{-2} are the association and dissociation rates, respectively, according to Scheme II. The slope of the line in Figure 7 gives $k_2 = 0.78 \pm 0.02$ μM^{-1} s^{-1}. The intercept provides an inaccurate estimate of $k_{-2} = 0.44 \pm 0.77$ s^{-1}; a better estimate of the dissociation rate can be calculated from the $K_{d,G}$ and the binding rate to be $k_{-2} = 0.12 \pm 0.02$ s^{-1}.

Rate of S3P Binding. The rate of S3P binding to EPSP synthase was measured by following the quenching of intrinsic enzyme fluorescence after mixing enzyme with varying S3P concentrations in the presence of a fixed concentration of glyphosate. According to Scheme II, the rate of S3P binding could be measured directly by following the time dependence of the fluorescence change if k_{-1} is small compared to $k_2[G]$.

At each concentration of S3P, the kinetic data followed a single exponential as shown in Figure 6. The dependence of the observed rate on the concentration of S3P fit a hyperbola (see Figure 8). These data suggest that the binding of S3P

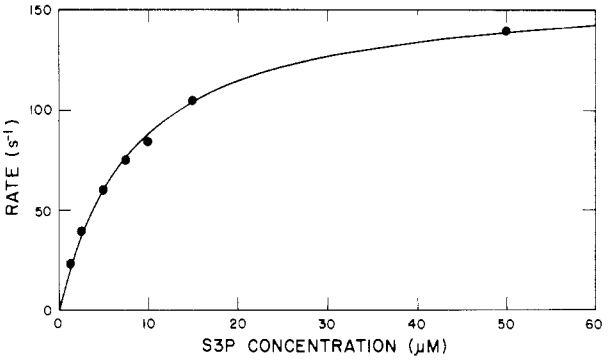

FIGURE 8: Concentration dependence of the rate of S3P binding. The rate of S3P binding to EPSP synthase was measured by following the quenching of intrinsic enzyme fluorescence in the presence of a fixed concentration of 200 μM glyphosate. The observed rate of binding then follows the equation $k_{obsd} = k_2[S][G]/([S] + K_{d,S})$. The curve represents the best fit by nonlinear regression analysis to the equation with $K_{d,S} = 8.5 \pm 0.5$ μM and $k_2[G] = 162 \pm 4$ s^{-1} ($k_2 = 0.8$ μM^{-1} s^{-1}).

is a rapid equilibrium such that k_{-1} is greater than $k_2[G]$. This conclusion is supported by the observation that the observed rate is independent of whether S3P is preincubated with the enzyme or added at the same time as glyphosate. Accordingly, the two-step binding process can be simplified to that shown in Scheme III. The concentration dependence of the observed rate of binding then follows a hyperbola:

$$k_{obsd} = k_2[S][G]/(K_{d,S} + [S]) + k_{-2} \quad (4)$$

The line drawn in Figure 8 represents the best fit by nonlinear regression analysis to eq 4 with $K_{d,S} = 8.5 \pm 0.5$ μM and $k_2[G] = 162 \pm 4$ s^{-1}; these values are in good agreement with the previous determinations of the dissociation constant for S3P and the rate of glyphosate binding, shown above. Although these data do not allow a precise determination of the rate of S3P binding, they do provide a lower limit of $k_1 \geq 2 \times 10^7$ M^{-1} s^{-1}, from the limiting slope at low S3P concentration.

Scheme III

$$E \underset{}{\overset{K_1[S]}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2[G]}{\rightleftharpoons}} E{\cdot}S{\cdot}G^*$$

We attempted to measure more accurately the rate of S3P binding by extending the rate measurements to higher glyphosate concentrations. As $k_2[G]$ increases and becomes greater than k_{-1}, the rate of the observed fluorescence transient should approach a limit set by the rate of S3P binding, $k_1[S]$ (Scheme II). An experiment was performed in which glyphosate (2 mM) and S3P (2 μM) were mixed in the stopped-flow apparatus with 1 μM enzyme (final concentrations) and the change in fluorescence was recorded over 40 ms (data not shown). Only a 2-fold excess of S3P over enzyme was used to maximize the signal (high enzyme concentration) and yet provide a rate within the time resolution of the instrument. Because the S3P concentration was only a 2-fold excess over enzyme, it would not be valid to fit the data to a single exponential. Rather, the data were analyzed by computer simulation using the program KINSIM (Barshop et al., 1983) to provide the best estimate for the binding rate as described under Materials and the Methods. This analysis indicated that $k_1 \geq 2 \times 10^8$ M^{-1} s^{-1}, and according, from the $K_{d,S}$, $k_{-1} \geq 1400$ s^{-1}.

Rate of EPSP Binding. The rate of EPSP binding proved difficult to measure due to a combination of the weak fluorescence signal observed with the stopped flow and the fast reaction rate. By use of the $K_{d,EPSP}$ measurement of 1.0 μM

and the turnover rate of 60 s^{-1}, lower limits have been set for both the binding rate ($\geq 60\ \mu M^{-1}\ s^{-1}$) and the dissociation rate ($\geq 60\ s^{-1}$).

Discussion

A summary of all equilibrium and kinetic data appears in Table I. Considering the potency of glyphosate as a herbicide, the binding of glyphosate to the enzyme–S3P complex is not as tight as one might have expected. The effectiveness of glyphosate can be attributed to two factors. First, because glyphosate binds in the presence of S3P, increasing concentrations of S3P resulting from the inhibition of the enzyme will augment, rather than overcome, the inhibition. Second, plant studies have shown that the transport of glyphosate into actively growing tissues contributes to it effectiveness (Cole, 1985).

EPSP binds 7-fold tighter than S3P; the higher affinity of the enzyme for EPSP could be the result of an ionic interaction between the carboxyl group of EPSP and a positively charged amino acid residue at the enzyme active site. This ionic interaction could account for the 0.9 kcal/mol difference in binding energy.

Data on the kinetics of S3P binding in the presence of glyphosate suggested a rapid equilibrium for S3P binding. Estimates of the S3P binding rate, obtained at high glyphosate concentrations, indicated that S3P dissociates at a rate greater than 1400 s^{-1}. This rate is fast enough to account for the observation of a single exponential in all of the measurements of the S3P binding rate and justify the fitting of the concentration dependence to a hyperbola (Figure 8).

Both S3P and EPSP appear to bind and dissociate rapidly; the observation that the Michaelis constant for each substrate is comparable to the dissociation constant supports the conclusion that the binding of S3P and that of EPSP occur as rapid equilibria, followed by the binding of the second substrate, PEP or P_i, respectively.

The manner in which glyphosate interacts with S3P and enzyme to form the tight ternary complex is subject to speculation. Because glyphosate has been shown to competitively inhibit PEP binding, it seems likely that the carboxylate and the phosphonate groups of glyphosate are binding to the carboxylate and phosphate portions of the PEP binding site on the enzyme, respectively. It is then tempting to speculate that the amino group of glyphosate interacts with an acidic group on the enzyme that serves to protonate the vinyl group of PEP during the reaction (Bondinell et al., 1971; Anton et al., 1983). Glyphosate could then be considered as a transition-state analogue mimicking a protonated carbonium ion species of PEP. However, there are other groups likely to be at the active site that could interact favorably with the amino group of glyphosate, and one must also consider the potential for interaction between glyphosate and S3P.

It has been suggested that a transition-state analogue should bind more tightly and more slowly than the substrate (Frieden et al., 1980). The dissociation constant for glyphosate is only 15-fold lower than the Michaelis constant for PEP. Although one cannot directly relate the Michaelis constant to the binding affinity for PEP, the current data would suggest only a small contribution to glyphosate binding from the utilization of energy normally involved in the stabilization of the transition state. The rate of PEP binding is similarly not known, but a minimum estimate can be obtained from $k_{cat}/K_m = 5\ \mu M^{-1}\ s^{-1}$, which is only slightly larger than the rate of glyphosate binding, $0.78\ \mu M^{-1}\ s^{-1}$. Although there is sufficient question concerning the general validity of the rule for slow binding of transition-state analogues, on the whole, the current data provide some support for the binding of glyphosate as a transition-state analogue.

There are two alternate hypotheses for the EPSP synthase enzyme reaction mechanism. One suggests a mechanism involving a covalent enolpyruvoyl-enzyme intermediate (Anton et al., 1983), while the other suggests a direct addition–elimination mechanism involving a tetrahedral intermediate resulting from the nucleophilic attack of the 5-hydroxyl group of S3P on carbon 2 of PEP (Bondinell et al., 1971). No definitive data exist to eliminate either mechanism although the published data have been taken as support for one mechanism or the other. The current work provides the data required to design and interpret the experiments to establish the pathway and identify the intermediate, with the ultimate goal of a complete kinetic and thermodynamic description of the reaction pathway (Anderson et al., 1988).

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Appendix: Derivation of the Apparent Dissociation Constant for Shikimate 3-Phosphate Binding in the Presence of Glyphosate

In the main text of this paper, an apparent dissociation constant was measured for S3P (S) binding in the presence of glyphosate (G). The K_d^{app} is a function of the intrinsic dissociation constants for S3P ($K_{d,S}$) and glyphosate ($K_{d,G}$) and the concentration of glyphosate. In this Appendix we present the derivation of an expression for K_d^{app} using one simplifying assumption.

We begin with ordered sequential binding of S3P and glyphosate

$$E + S \rightleftharpoons ES \qquad K_{d,S} = \frac{[E][S]}{[ES]} \tag{A-1}$$

$$ES + G \rightleftharpoons ESG \qquad K_{d,G} = \frac{[ES][G]}{[ESG]} \tag{A-2}$$

and the mass balance equations:

$$\begin{aligned} [E_0] &= [E] + [ES] + [ESG] \\ [S_0] &= [S] + [ES] + [ESG] \\ [G_0] &= [G] + [ESG] \end{aligned} \tag{A-3}$$

The fluorescence change occurs with the formation of ESG while ES is silent. Therefore, the equation describing the formation of ESG from E can be obtained by combining eq A-1 and A-2:

$$K_{d,S}K_{d,G} = \frac{[E][S][G]}{[ESG]} \tag{A-4}$$

If $[G_0] \gg [E_0]$, then [G] can be assumed to be constant, $[G] \approx [G_0]$. The titration is carried out under conditions where $[G_0] \gg K_{d,G}$ so that the ratio of [ESG]/[ES] is high and the concentration of ES is then negligible relative to E and ESG. Accordingly

$$\begin{aligned} [E] &\approx [E_0] - [ESG] \\ [S] &\approx [S_0] - [ESG] \end{aligned} \tag{A-5}$$

The apparent dissociation constant is then defined by

$$K_{d,S}^{app} \equiv \frac{[E][S]}{[ESG]} = \frac{K_{d,S}K_{d,G}}{[G_0]} \tag{A-6}$$

Substitution of eq A-5 into eq A-6 yields the quadratic

$$[ESG] = \{(K_d^{app} + [E_0] + [S_0]) - \sqrt{(K_d^{app} + [E_0] + [S_0])^2 - 4[E_0][S_0]}\}/2 \quad (A-7)$$

Of course, if $[S_0] \gg [E_0]$, then the solution reduces to a hyperbola:

$$[ESG] = \frac{[E_0][S_0]}{K_d^{app} + [S_0]} \quad (A-8)$$

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Deuterium Isotope Effects in Norcamphor Metabolism by Cytochrome P-450$_{cam}$: Kinetic Evidence for the Two-Electron Reduction of a High-Valent Iron–Oxo Intermediate

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ABSTRACT: The kinetics of NADH consumption, oxygen uptake, and hydrogen peroxide production have been studied for norcamphor metabolism by cytochrome P-450$_{cam}$. The kinetic deuterium isotope effects on these processes, with specifically deuteriated norcamphor, are 0.77, 1.22, and 1.16, respectively. Steady-state UV–visible spectroscopy indicates that transfer of the second electron to the dioxy ferrous P-450 is the rate-limiting step, as it is when camphor is the substrate. The inverse deuterium isotope effect for NADH consumption is consistent with an isotope-dependent branching between monooxygenase and oxidase activity, where these reactivities differ in their NADH:oxygen stoichiometries. However, no isotope-dependent redistribution of steady-state intermediates was detected by isotopic difference UV–visible spectroscopy in the presence of norcamphor. The kinetic isotope effects and steady-state spectral results suggest that the high-valent iron–oxo hydroxylating intermediate $[FeO]^{3+}$ is reduced by NADH and the physiological electron-transfer proteins to afford water.

The four-electron reduction of molecular oxygen to form 2 equiv of water is traditionally ascribed to non-heme, copper-containing oxidases (Malmstrom et al., 1975), copper-containing monooxygenases (Hamilton et al., 1972), and cytochrome oxidase, a structurally complex two-heme and two-copper enzyme (Caughey et al., 1976; Poynton & Schatz, 1975). It also has been suggested that this type of oxidase activity occurs with various cytochrome P-450's (Staudt et al., 1974; Zhukovr & Archakov, 1982), and recently it was demonstrated that several purified hepatic P-450's catalyze this reaction without reduction of intermediate hydrogen peroxide, although peroxide formation was detected in all the isozymes studied (Gorsky et al., 1984). Recently, we reported that, in the presence of the substrate analogue norcamphor, the soluble cytochrome P-450$_{cam}$, isolated from *Pseudomonas putida*, exhibits an efficient four-electron oxidase activity in addition to the expected monooxygenation of substrates (Atkins & Sligar, 1987). Hydrogen peroxide formation was minimal compared to water production in this complete reconstituted system containing P-450$_{cam}$, putidaredoxin, putidaredoxin reductase, and NADH. Furthermore, a deuterium isotope effect on the ratio of monooxygenase/oxidase activity was observed, which led to the proposal of a common intermediate for both activities. Since chemical steps of the P-450 reaction cycle subsequent to formation of the dioxy ferrous intermediate ($P\text{-}450^{rs}_{O_2}$)[1] demonstrate a strong commitment to catalysis and are expected to be irreversible (Harada et al., 1984), and since the only isotopically sensitive step would be hydrogen (deu-

[1] Abbreviations: $P\text{-}450^{rs}_{O_2}$, dioxy ferrous form of P-450; Pd, putidaredoxin; Fp, putidaredoxin reductase; $(V_{max})_{O_2}$, steady-state velocity of oxygen consumption with saturating levels of the appropriate substrate; $(V_{max})_{H_2O_2}$, steady-state velocity of hydrogen peroxide production with saturating levels of these substrates; $(V_{max})_{NADH}$, corresponding steady-state velocity of NADH oxidation; I, *exo,exo*-norcamphor-*5,6-d*$_2$; II, *exo,exo*-norcamphor-*3,3,5,6-d*$_4$; Tris, tris(hydroxymethyl)aminomethane; GC, gas chromatography.

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