EPSP Synthase: Binding Studies Using Isothermal Titration Microcalorimetry and Equilibrium Dialysis and Their Implications for Ligand Recognition and Kinetic Mechanism‡

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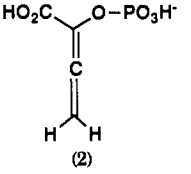
ABSTRACT: Isothermal titration calorimetry measurements are reported which give important new binding constant (K_d) information for various substrate and inhibitor complexes of *Escherichia coli* EPSP synthase (EPSPS). The validity of this technique was first verified by determining K_d's for the known binary complex with the substrate, shikimate 3-phosphate (S3P), as well as the herbicidal ternary complex with S3P and glyphosate (EPSPS·S3P·glyphosate). The observed K_d's agreed very well with those from previous independently determined kinetic and fluorescence binding measurements. Further applications unequivocally demonstrate for the first time a fairly tight interaction between phosphoenolpyruvate (PEP) and free enzyme (K_d = 390 μM) as well as a correspondingly weak affinity for glyphosate (K_d = 12 mM) alone with enzyme. The formation of the EPSPS·PEP binary complex was independently corroborated using equilibrium dialysis. These results strongly suggest that S3P synergizes glyphosate binding much more effectively than it does PEP binding. These observations add important new evidence to support the hypothesis that glyphosate acts as a transition-state analogue of PEP. However, the formation of a catalytically productive PEP binary complex is inconsistent with the previously reported compulsory binding order process required for catalysis and has led to new studies which completely revise the overall EPSPS kinetic mechanism. A previously postulated ternary complex between S3P and inorganic phosphate (EPSPS·S3P·P_i, K_d = 4 mM) was also detected for the first time. Quantitative binding enthalpies and entropies were also determined for each ligand complex from the microcalorimetry data. These values demonstrate a clear difference in thermodynamic parameters for recognition at the S3P site versus those observed for the PEP, P_i, and glyphosate sites.

The enzyme EPSP[1] (5-enolpyruvoylshikimate-3-phosphate) synthase (EPSPS, EC 2.5.1.19) has been the subject of ongoing investigations as a target for mechanism-based inhibitor design [for reviews, refer to Sikorski et al. (1991) and Anderson and Johnson (1990a)]. EPSPS functions as the biological target in plants for glyphosate (*N*-phosphonomethylglycine), the active ingredient in the broad spectrum herbicide Roundup (Amrhein et al., 1980; Franz, 1985). This enzyme catalyzes an unusual transfer reaction of the carboxyvinyl group from phosphoenolpyruvate (PEP) regiospecifically to the 5-OH of shikimate 3-phosphate (S3P) to form EPSP as shown in Scheme I.

The EPSPS chemical mechanism proceeds through a single, kinetically competent tetrahedral intermediate (**1**) (Anderson et al., 1988a). This intermediate has been isolated (Anderson et al., 1988b) and binds tightly upon exposure to enzyme (Anderson & Johnson, 1990b). Several classes of structurally related bisubstrate inhibitors have been synthesized which act as potent inhibitors by simultaneously occupying both the S3P and PEP sites (Alberg & Bartlett, 1989; Pansegrau et al., 1991). Access to the PEP-phosphate cavity appears to be a key requirement for tight-binding inhibition; yet little information has been available to describe details of this critical interaction. Similarly, nothing is known about the availability of the glyphosate and PEP binding pockets in native enzyme in the absence of S3P.

EPSPS catalysis (Anderson et al., 1988a) and glyphosate inhibition (Boocock & Coggins, 1983; Steinrücken & Amrhein, 1984) reportedly proceed through a compulsory ordered binding process where both PEP and glyphosate bind preferentially to the preformed EPSPS·S3P binary complex. Recent studies have more clearly defined the structural and ionic requirements for glyphosate (Ream et al., 1988) and PEP (Walker et al., 1991) binding in the presence of S3P. Kinetic and ^{31}P NMR spectral characterizations of several PEP analogue inhibitors suggest that both carboxyallenyl phosphate (**2**) and (*Z*)-3-fluoro-PEP (**3**) have significant binding affinity



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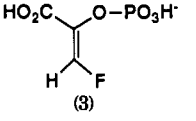

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for free EPSPS as well as the EPSPS·S3P binary complex (Walker et al., 1991; Gruys et al., 1992).

Several unsuccessful attempts have been made using gel filtration, NMR (Castellino et al., 1989), and fluorescence (Anderson et al., 1988c) methods to detect some affinity for either PEP or glyphosate alone with free enzyme. Each of these techniques is severely limited in its ability to detect weakly bound complexes. Recently, methodology based on isothermal titration calorimetry has developed sufficiently to

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[1] Abbreviations: EPSPS, 5-enolpyruvoylshikimate-3-phosphate synthase; EPSP, 5-enolpyruvoylshikimate 3-phosphate; glyphosate, *N*-(phosphonomethyl)glycine; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; S3P, shikimate 3-phosphate.

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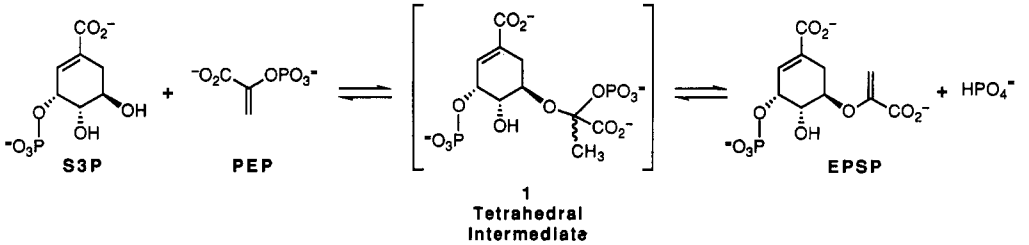
Scheme I



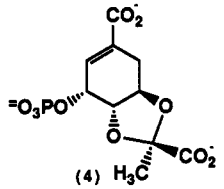
examine enzyme–substrate complexes (Wiseman et al., 1989; Freire et al., 1990). This technique offers distinct advantages over previous methods by allowing the direct detection of relatively weak ($K_d \sim$ millimolar) enzyme–ligand complexes and also provides quantitative measurements of the enthalpies and entropies of binding, when sufficient quantities of pure enzyme are available to utilize this method.

Here we report the results of microcalorimetry and equilibrium dialysis studies with overexpressed *Escherichia coli* EPSPS using PEP, glyphosate, and inorganic phosphate alone and in combination with S3P. These results demonstrate for the first time that PEP has a reasonable affinity for free enzyme, relative to glyphosate. These findings have important implications for the overall EPSPS kinetic mechanism (Gruys et al., 1992) and provide additional evidence that glyphosate functions as a transition-state analogue of PEP as originally proposed by Steinrücken and Amrhein (1984).

Materials and Methods

Enzyme. EPSP synthase was purified from a strain of *E. coli* (pMON6001) overexpressing EPSPS (Rogers et al., 1983) as described previously (Castellino et al., 1989). The enzyme concentration was determined using an extinction coefficient of 35 200 M^{-1} cm^{-1} at 280 nm.

Chemicals. S3P was prepared enzymatically as the sodium salt from shikimic acid (Sigma) using shikimate kinase (Millar et al., 1986) as described previously (Castellino et al., 1991). [U-^{14}C]S3P was prepared similarly from uniformly labeled [^{14}C]shikimic acid (New England Nuclear) as described by Padgette et al. (1987). Analytical grade glyphosate (purity >99%) and [^{14}C]glyphosate (1 mM, 8 mCi/mmol) were obtained from internal Monsanto stocks. PEP was obtained as the potassium salt (Sigma). [2-^{14}C]PEP was obtained from Amersham (0.604 mM, 35.7 μCi/mmol). The EPSP ketal (**4**) was obtained from Dr. R. Douglas Sammons (Leo et al.,



1990). Inorganic phosphate was used as the potassium salt. All other buffers and reagents employed were analytical grade and obtained commercially.

Microcalorimetry. All microcalorimetry experiments were carried out in binding buffer (50 mM Hepes/KOH, 50 mM potassium chloride, pH 6.8) degassed by stirring under vacuum. For experiments requiring high enzyme concentrations (1 mM), 2-mercaptoethanol (5 mM) was added to this buffer. Purified EPSPS was exchanged into degassed binding buffer through repeated concentration by ultrafiltration (Amicon, YM10 membrane) and resuspension in binding buffer. The enzyme concentration was adjusted by A_{280} using an extinction coefficient of 35 200 M^{-1} cm^{-1}. Ligands were prepared in binding buffer, and the pH was adjusted to 6.8 if necessary. Enzyme and ligand solutions were degassed under vacuum prior to analysis.

Ligand binding to EPSPS was analyzed in a Microcal Omega titration calorimeter equilibrated to 27 °C. The sample and reference cells of the calorimeter were previously filled completely with degassed enzyme solution and deionized water, respectively. In a typical experiment, 20 5-μL aliquots of ligand solution were injected into about 1.4 mL of stirring (425 rpm) enzyme solution for a duration of 18 s with 3 min between injections. The heats of reaction were determined by integration of the peak observed. To correct for ligand heats of dilution, a control experiment was also performed using similar conditions with solutions containing buffer but no enzyme. After the contribution from the heat of dilution of each injection was subtracted, the sum of the heat evolved was plotted against the total ligand concentration to produce the binding isotherm. Binding constants, heats of binding, and stoichiometry were determined by fitting the binding isotherm against the binding equation described by Freire et al. (1990) for ligand binding to a macromolecule possessing one set of independent ligand-binding sites. The data were deconvoluted using a nonlinear least-squares algorithm incorporated in the Omega instrument software as described by Wiseman et al. (1989).

Careful sample preparation was required for all of the microcalorimetry experiments. Both titrants and enzyme samples had to be in exactly the same buffer, at the same pH, and degassed immediately before analysis to reduce errors associated with these factors.

Equilibrium Dialysis. Purified, *E. coli* EPSPS was dialyzed at 5 °C into dialysis buffer [50 mM Hepes/KOH, 100 mM KCl, 10%(w/v) glycerol, 5 mM 2-mercaptoethanol, pH 7.0]. The experiments were performed by dialyzing a constant amount of enzyme against increasing concentrations of ^{14}C-labeled ligand. Enzyme (200 μL) was placed into one half-cell of a Hoeffer Microdialyzer (Hoeffer Scientific) equipped with a dialysis membrane (12 000–14 000 MW cutoff, Hoeffer Scientific), and an equal volume of ligand solution was placed in the other half-cell. Dialysis cells were rotated (20 rpm) for approximtely 16 h at 5 °C. Radioactivity in 100-μL aliquots was determined by liquid scintillation counting (Beckman LS). Free and bound ligand concentrations were calculated from the amount of radioactivity present in the half-cell containing protein (= bound plus free ligand) and the half-cell without protein (= free ligand). Data were then plotted as $1/[\text{ligand}]_{\text{free}}$ versus $[\text{EPSPS}]/[\text{ligand}]_{\text{bound}}$ to determine the equilibrium dissociation constant (K_d) and stoichiometry (n) (Klotz et al., 1946).

Results

Characterization of Enzyme–Ligand Complexes. Isothermal titration microcalorimetry takes advantage of changes in heat flow that accompany ligand binding. Thus, the amount of heat released or absorbed during the addition of ligand to enzyme is directly proportional to the overall change in the

Table I. Characterization of Select EPSP Synthase Binary and Ternary Ligand Complexes by Microcalorimetry at 27 °C[a]

complex	K_d (μM)	stoichiometry (n)	ΔH (kcal/mol)	ΔS (cal/mol·K)
(a) enz + S3P	10 ± 0.7	1.03 ± 0.02	−5.2 ± 0.1	5.4 ± 0.6
(b) enz + EPSP ketal	24 ± 8	1.21 ± 0.02	−1.4 ± 0.01	16.4 ± 0.8
(c) enz + PEP	390 ± 15	0.69 ± 0.10	−4.9 ± 0.3	−0.6 ± 0.8
(d) enz + glyphosate	12000 ± 2000	1.00[b] ± 0.0	−13.7 ± 1.6	−36.7 ± 5.5
(e) enz·S3P + glyphosate	0.15 ± 0.03	0.99 ± 0.02	−19.0 ± 0.4	−31.9 ± 1.8
(f) enz·S3P + P_i	4000 ± 300	1.67 ± 0.28	−5.5 ± 1.0	−7.5 ± 3.4
(g) enz·ketal + P_i	13200 ± 1600	1.00[b] ± 0.0	−15.2 ± 1.4	−42.2 ± 4.9

[a] Values in this table represent the average value from at least duplicate titration experiments performed using the same sample solutions on the same day. Titrant (on right side of the complex column) was injected sequentially into stirring sample solution (on left side of the complex column). The sample cell contained EPSPS ("enz") in buffer for characterization of binary complexes, or it contained a binary complex (formed by a 10-fold excess of ligand over enzyme concentration) for the evaluation of ternary complexes. Enzyme and ligand concentrations used for each complex are as follows: (a) 0.3 mM EPSPS and 6 mM S3P; (b) 0.5 mM EPSPS and 12.5 mM EPSP ketal; (c) 1 mM EPSPS and 25 mM potassium phosphate; (d) 1 mM EPSPS and 25 mM glyphosate; (e) 0.09 mM EPSPS, 0.9 mM S3P, and 2 mM glyphosate; (f) 1 mM EPSPS, 10 mM S3P, and 25 mM potassium phosphate; and (g) 1 mM EPSPS, 10 mM EPSP ketal, and 25 mM potassium phosphate. [b] The stoichiometry was fixed at 1.00 to begin data analysis. After optimization of K's and ΔH, the stoichiometry was allowed to float leading to these values.

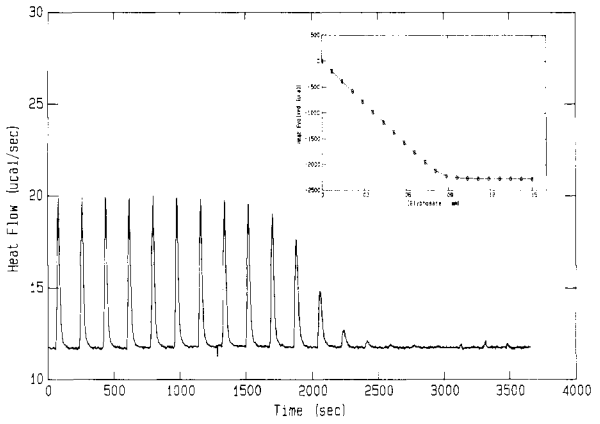


FIGURE 1: Titration isotherm of glyphosate binding to preformed EPSPS·S3P complex at 27 °C. 5-μL aliquots of 2.0 mM glyphosate were injected in each step into 1.4 mL of 0.09 mM EPSPS containing 0.9 mM S3P at pH 6.8. (Insert) Binding curve showing total heat evolved versus cumulative concentration of added glyphosate. The curve was calculated from the binding equation described by Freire et al. (1990), and data were fitted using a nonlinear least-squares algorithm (Wiseman et al., 1989) supplied with the Microcal Omega instrument software.

concentration of bound ligand. Typically, a solution of titrant is sequentially injected into a stirring cell containing enzyme sample, and heat flow from each injection is measured over time. A representative titration series is shown in Figure 1. Aliquots of glyphosate were injected into a stirring cell containing preformed EPSPS·S3P binary complex until little heat is detected at high total concentrations of glyphosate. From this single binding isotherm, the dissociation constant (K_d) and number of binding sites (stoichiometry, n), as well as the enthalpic and entropic contributions to the Gibbs free energy of association ($\Delta G = \Delta H - T\Delta S$), can be determined as described under Materials and Methods. As apparent in Figure 1, an excellent time response is observed following each injected aliquot.

This type of analysis was applied to the investigation of several EPSPS substrate and inhibitor complexes. The results are summarized in Table I. The physical parameters describing enthalpies and entropies of association are also listed in Table I for each of the EPSPS complexes examined. Four binary and three ternary EPSPS complexes were characterized by isothermal titration microcalorimetry. To validate the method, the known EPSPS·S3P binary and EPSPS·S3P·glyphosate ternary complexes were characterized for comparison with previous kinetic (Boocock & Coggins, 1983; Steinrücken & Amrhein, 1984) and fluorescence binding data (Anderson et al., 1988c). The observed K_d for formation of the EPSPS·S3P binary complex using microcalorimetry was 10 ± 0.7 μM, in very good agreement with the previously reported 7 ± 1.2 μM K_d from competitive fluorescence measurements. Similarly, binding of glyphosate to the preformed EPSPS·S3P complex gave an observed K_d of 0.15 ± 0.03 μM by microcalorimetry for the resulting ternary complex, in excellent agreement with previous kinetic (K_i = 0.16 μM) and fluorescence (K_d = 0.16 ± 0.02 μM) data. Having established confidence in the approach from these results, we pursued additional binding data for several previously unreported EPSPS complexes to probe the limitations, scope, and utility of this technique, particularly with weaker ligands.

P_i Binding to EPSPS Complexes. Recently, several classes of bisubstrate EPSPS inhibitors have been identified as close structural analogues of intermediate **1**. Access to the PEP-phosphate site is known to contribute significantly to the potency of both shikimate and aromatic (Alberg & Bartlett, 1989; Pansegrau et al., 1991) inhibitors, but little is known about this relatively weak (K_m = 2.5 mM) binding domain (Boocock & Coggins, 1983; Duncan et al., 1984). The EPSPS·S3P·P_i ternary complex has been previously postulated to exist on the basis of rapid-quench kinetic experiments (Anderson et al., 1988a), but it has not been detected directly. Binding of P_i to the preformed EPSPS·S3P binary complex gave an observed K_d of 4 ± 0.3 mM for the resulting ternary complex, slightly weaker than the fitted K_d value of 0.7 mM predicted from the overall reported kinetic model (Anderson et al., 1988a).

The EPSP ketal (**4**) is a novel EPSP synthase inhibitor produced in an enzymatic side reaction off the normal catalytic path (Leo et al., 1990). Presumably, formation of this ketal relies on an S_N2 displacement of P_i by the shikimate 4-OH in **1**. Thus, binding of P_i should be permitted in the presence of the EPSP ketal. Formation of the EPSPS·EPSP ketal binary complex was observed to occur by microcalorimetry with a K_d of 24 ± 8 μM. This result is consistent with the relatively moderate competitive inhibition activity observed for this compound (K_i = 32 μM) versus S3P (Sammons et al., unpublished results). Some weak binding of P_i (K_d = 13.2 ± 1.6 mM) to the preformed EPSPS·EPSP ketal complex also could be detected. This result suggests that the P_i site is accessible at the active site in the presence of the EPSP ketal but is sterically perturbed to a weaker interaction versus that observed wtih S3P. In each of these cases, P_i binding appears slightly weaker than the catalytic P_i complex formed with EPSP (K_m = 2.5 mM) for the enzymatic reverse reaction (Duncan et al., 1984).

An unambiguous determination of absolute stoichiometry was difficult for these weak P_i complexes. The observed K_d

values were at the upper limit of the practical working range (1×10^{-2} M to 1×10^{-8} M) of this instrument. For the EPSPS·S3P·P_i ternary complex, a best fit of the data was achieved with the stoichiometry preset for two, but a good fit could also be obtained using a stoichiometry fixed at one. Thus, the data could not determine unambiguously how many P_i binding sites are available when S3P is bound to EPSPS. However, the ability to detect these weaker (millimolar) EPSPS–ligand complexes prompted us to explore the interaction of PEP and glyphosate alone with enzyme.

PEP Binding to EPSPS Complexes. As shown in Table I, PEP will form a reasonably tight EPSPS binary complex with an observed K_d of 390 ± 15 μM. The ability of PEP to bind to free enzyme is therefore perturbed only by approximately 20-fold relative to the observed K_m (19 μM) in the presence of S3P (Anderson et al., 1988a). This was a surprising and completely unexpected result given multiple unsuccessful literature reports to establish this interaction between PEP and free enzyme. Presumably, PEP binding to native EPSPS occurs at its catalytic site. The formation of a productive EPSPS·PEP binary complex is clearly not consistent with previous kinetic studies suggesting that EPSPS catalysis requires a compulsory binding order process with S3P binding first (Anderson et al., 1988a; Anderson & Johnson, 1990a). Indeed, recent steady-state kinetic investigations corroborate the observed affinity of ~400 μM for PEP binding to free enzyme in a catalytically productive complex and revise the overall kinetic reaction mechanism to a random process with synergistic binding between substrates (Gruys et al., 1992).

This unexpected finding led us to seek additional corroboration of the PEP binary data using an alternative binding technique. A similar affinity of PEP for free enzyme (K_d = 400 ± 60 μM) can also be demonstrated using equilibrium dialysis methods, although the observed stoichiometry is slightly higher (n = 1.4) than that obtained by microcalorimetry. Once again, formation of the EPSPS·S3P binary complex (K_d = 10 ± 3.1 μM) was used as a standard to establish the validity of this EPSPS equilibrium dialysis procedure.

Glyphosate Binding to EPSPS Complexes. On the basis of the above results, we also investigated the affinity of glyphosate for free enzyme. As before, microcalorimetry methods clearly demonstrated that glyphosate has a weak, yet detectable, interaction with free enzyme. The observed K_d for the EPSPS·glyphosate binary complex is 12 ± 2 mM (Figure 2B), significantly weaker than that observed for EPSPS·PEP (Figure 2C). The binding curve observed for the EPSPS·glyphosate bindary complex is fairly flat and near the practical limit described by Wiseman et al. (1989) for obtaining accurate K_d information. Nevertheless, the observed data fit the calculated binding curve quite well, and reasonable precision was obtained in triplicate experiments using 1mM EPSPS. On the basis of such weak binding, we were also concerned that perhaps some nonspecific ionic interaction might be occurring, particularly between glyphosate and EPSPS.

Glyphosate is well-known as a competitive inhibitor with respect to PEP (Boocock & Coggins, 1983; Steinrücken & Amrhein, 1984). Consequently, these kinetic results strongly suggest that significant overlap occurs between the PEP and glyphosate binding sites under catalytic conditions when S3P is present. If this common overlap between the glyphosate and PEP binding domains is retained in native enzyme, one species should compete with binding of the other. This phenomenon was investigated by evaluating the effect upon PEP binding

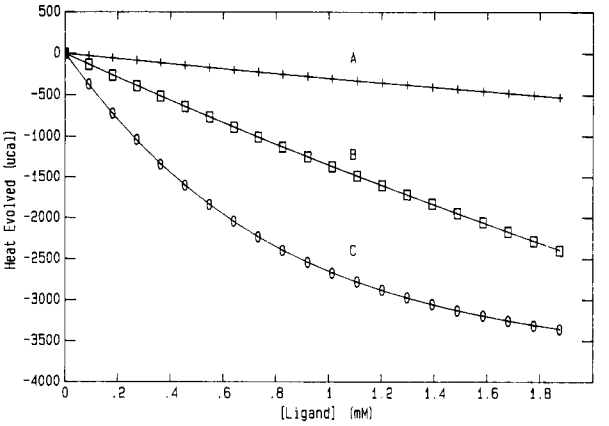


FIGURE 2: Competitive binding of glyphosate and PEP with native EPSP synthase at 27 °C. (A) Addition of 25 mM PEP to 1 mM EPSPS containing 100 mM glyphosate, pH 6.8 (+). (B) Addition of 96 mM glyphosate to 1 mM EPSPS, pH 6.8 (□); formation of EPSPS·glyphosate. (C) Addition of 25 mM PEP to 1 mM EPSPS, pH 6.8 (○); formation of EPSPS·PEP. The curves were calculated from the binding equation described by Freire et al. (1990), and data were fitted using a nonlinear least-squares algorithm (Wiseman et al., 1989) supplied with the Microcal Omega instrument software.

when EPSPS was preequilibrated with a 100-fold excess of glyphosate. By microcalorimetry, preequilibration of enzyme with glyphosate to form the now established EPSPS·glyphosate binary complex markedly reduced the total amount of heat released upon titration with PEP (Figure 2A), supporting interaction at similar enzyme sites. Glyphosate also weakly inhibits (K_i ~ 12 mM) the turnover of shikimate as an alternate substrate. In this reaction, a change in kinetic mechanism occurs (Gruys et al., 1992) where PEP now binds first. Under these conditions, glyphosate also binds competitively with PEP for free enzyme.

The small amount of residual PEP binding observed under these conditions suggests that PEP can still weakly bind at another enzyme site. A rigorous data analysis was not possible for this complex based on the low ΔH and weak binding observed. A preliminary analysis using a stoichiometry fixed at one gives a residual K_d for PEP of approximately 10 mM in the presence of saturating glyphosate. Recent steady-state kinetic studies verify the substrate inhibition properties of PEP in a dead-end 2:1 complex, presumably through a weak interaction at the S3P site (Gruys et al., 1992). The steady-state kinetic data indicate that PEP can bind at the S3P site with an observed affinity of 6–8 mM. Thus, the competitive microcalorimetry data (Figure 2) are consistent with the kinetic results but are not sufficient to specify the site of interaction. The same competitive phenomenon was also observed by equilibrium dialysis where PEP affinity to native EPSPS was reduced 95% by the presence of a 100-fold excess of glyphosate in the enzyme solution.

Discussion

The major advantage of titration microcalorimetry is that the binding isotherms are defined by the heat of the reaction such that the energetics of binding are determined as well as the association (or dissociation) constant (Eftink & Biltonen, 1980). The thermodynamic description of ligand binding with enzymes has been reviewed by Eftink and Biltonen (1980) as well as by Fersht (1985). Besides contributions from coupled equilibria, such as those involving release or desorption of protons by the buffer and changes in the state of aggregation of either component, the observed thermodynamic changes can be due to a combination of (a) changes in solvation state of

the ligand or protein, (b) complementary interaction between macromolecule and ligand at the binding site through hydrogen bonds, van der Waals forces or hydrophobic, dipole–dipole, and electrostatic interactions, and (c) changes in macromolecular conformations induced by ligand binding.

While it is currently not feasible to predict the enthalpies and entropies of enzyme–ligand interactions, the magnitude and the sign of the enthalpy or entropy changes are sensitive diagnostic tools for the structural changes that can accompany ligand recognition. Previous calorimetry studies of ligand association with multiply charged anions such as phosphate (Kuriki et al., 1976) and mono-, di-, or trinucleotides (Hinz, 1983) demonstrate that there are no clearly predictive trends across different enzymes for these thermodynamic parameters. Each enzyme/ligand system must be considered independently. In view of the many possible contributions associated with ligand binding, an exact assessment of the thermodynamic changes observed for EPSPS is not possible. However, qualitative comparisons of the enthalpies and entropies of binding from Table I for various EPSPS complexes dramatically illustrate the interplay that can occur between these parameters during ligand binding. Careful examination of the observed values in Table I indicates that there are dramatic differences influencing recognition at the S3P site versus those occurring at the PEP, P_i, and glyphosate sites.

S3P and EPSP Ketal Binary Complexes. The formation of these simple binary complexes helps illustrate anion recognition at the S3P binding site. The association of the EPSP ketal with enzyme is the most entropically favored complex observed with an accompanying ΔH near zero. This is typical for an association governed largely by a change in solvation state through release of bound water from the protein or ligand to the bulk solvent induced by either electrostatic or hydrophobic interactions (Eftink & Biltonen, 1980). Presumably, recognition of the EPSP ketal occurs through favorable electrostatic interactions at the anionic centers, particularly since the shikimate 3-phosphate group is known to contribute significantly (>8 kcal/mol) to recognition at the EPSPS active site (Gruys et al., 1992). The hindered cyclic ketal functionality should dramatically reduce any hydrogen-bond interactions donated by enzyme to either the 4- or 5-OH groups.

Similarly, recognition of S3P is also favorable in terms of the entropy term, but this effect is modulated through an enthalpy effect. In this case, again electrostatic interactions would be expected to predominate, but both enzyme and S3P can donate hydrogen bonds to stabilize the complex. Such hydrogen-bonding interactions generally contribute about 2 kcal/mol to the enthalpy term for each hydrogen bond with an accompanying decrease in entropy (Eftink & Biltonen, 1980).

PEP and Glyphosate Binary Complexes. The similarities in recognition at the shikimate sites for S3P and the EPSP ketal stand in sharp contrast to the differences in themodynamic parameters observed between PEP and glyphosate in their respective binary complexes. Recognition of PEP is largely enthalpy driven with an essentially zero entropy term. These characteristics are usually attributed to a conformational change in the macromolecule induced by substrate, although no corroborating evidence for a conformational change in EPSPS induced by PEP has yet been observed. Crystals of native *E. coli* EPSPS will disorder in the presence of PEP (Stallings et al., unpublished results). Interestingly, the observed enthalpy changes for PEP and S3P recognition are quite similar, but PEP recognition is attenuated by a more negative entropy effect.

The surprisingly tight interaction of PEP with free EPSPS elucidated in this study is not consistent with the compulsory-ordered mechanism proposed (Anderson et al., 1988a; Anderson & Johnson, 1990a) for catalysis, if this PEP binary complex is catalytically productive. A recent steady-state kinetic study now provides compelling evidence for an EPSPS random kinetic mechanism with synergistic binding between substrates (Gruys et al., 1992).

Glyphosate recognition in the binary complex is more favorable in terms of enthalpy effects than PEP but is destabilized by a very dramatic negative entropy term. The observed entropy loss is near the theoretical limit of 35 cal/(mol·K) normally attributed to a complete restriction in motional freedom of a bound ligand (Eftink & Biltonen, 1980). This large negative entropy term suggests that there is a high degree of complementarity between glyphosate and enzyme, with considerable restricted motion of side chains in this binary complex. Both EPSPS·PEP and EPSPS·glyphosate binary complexes were characterized and shown to have overlapping binding domains using competition experiments with microcalorimetry (Figure 2) and equilibrium dialysis.

P_i Ternary Complexes. Comparison of the thermodynamic parameters in these complexes is difficult based on the ambiguous stoichiometry measurements for the EPSPS·S3P·P_i system. The observed parameters for phosphate binding to EPSPS are quite different from those reported for phosphate binding to RNAse A (Fersht, 1985). The availability of multiple P_i binding sites in the presence of S3P is not inconsistent with the large number of positively charged residues encircling the EPSPS active site as recently established by X-ray crystallography of the native *E. coli* enzyme (Stallings et al., 1991). However, this capability to quantitate the interaction of free P_i with EPSPS, presumably at the PEP-phosphate site, now provides an interesting opportunity to evaluate novel bisubstrate inhibitors for their ability to exclude interaction at the PEP-phosphate site. Strong interaction with this site is known to be critical to achieving tight-binding EPSPS inhibitors (Alberg & Bartlett, 1989; Pansegrau et al., 1991).

The Herbicidal Glyphosate Ternary Complex with S3P. Formation of the ternary complex between glyphosphate and EPSPS·S3P is strongly driven by enthalpy much more than the EPSPS·glyphosate binary complex. However, a fairly large negative entropy term still counteracts this ligand association. These thermodynamic properties with such simple ligand structures are generally associated with a significant change in protein conformation during ligand association. Previous fluorescence binding studies strongly suggest that such a change in EPSPS conformation accompanies formation of the glyphosate ternary complex (Anderson et al., 1988c). The blue-shift accompanying ligand binding is consistent with a net increase in the amount of hydrophobic residues exposed to water. Presumably, the combined recognition of S3P and glyphosate at the EPSPS active site effectively neutralizes much of the positive charge in the protein. Recent crystallization results provide further corroboration by demonstrating that the glyphosate ternary complex crystallizes under completely different solvent conditions than the native *E. coli* enzyme (Stallings et al., unpublished results).

The large negative entropy term suggests that glyphosate fits extremely well within its binding site. Presumably, a series of electrostatic, dipole–dipole, and hydrogen-bonding interactions serve to restrict the motional freedom of glyphosate and many amino acid side chains in this complex. While a change in solvation state may also accompany glyphosate

binding to the EPSPS·S3P complex, any related overall positive entropy effect appears to be completely masked by the loss in motional freedom induced by glyphosate. Model studies suggest an unfavorable loss of nearly 4.3 entropy units per restricted side chain following ligand binding (Fersht, 1985). The multiple contributions of glyphosate functionalities in providing these interactions become apparent by comparing these results with the EPSPS·S3P·P_i complex. The large difference in ΔH observed for the glyphosate ternary complex suggests that the interactions between glyphosate and enzyme reach well beyond the phosphate site. This is consistent with the known restrictions in glyphosate structural specificity observed with other analogues (Ream et al., 1988) and may explain the greater thermal and proteolytic stability of this ternary complex over native enzyme.

Similarly, the thermodynamic parameters observed for formation of the EPSPS·S3P·glyphosate ternary complex represent the sum of the parameters for formation of the individual EPSPS·S3P and EPSPS·glyphosate binary complexes. This suggests that the formation of the ternary complex is an equilibrium process which can be approached through either of the individual binary complexes. This implies that the glyphosate site is at least partially exposed in native enzyme and that S3P helps organize the optimum glyphosate binding site. A significant interaction between S3P and enzyme as well as between S3P and glyphosate also may accompany the formation of the ternary complex. These results are consistent with those obtained using ^{31}P NMR methods which demonstrate that a dramatic change in both S3P *and* glyphosate chemical shifts occurs during ternary complex formation (Castellino et al., 1989).

A variety of biochemial and physiological evidence now points to the EPSPS·S3P·glyphosate ternary complex as the herbicidally relevant species at the molecular level (Sikorski et al., 1991). A comparison of the binding affinities for PEP and glyphosate alone with enzyme versus those achieved in the presence of S3P reveals that S3P synergizes glyphosate binding much more efficiently than PEP. Glyphosate has an approximately 75 000 times higher affinity for the EPSPS·S3P complex over free enzyme. In sharp contrast, PEP has only about a 20-fold higher affinity for the S3P-bound form. This substantially higher S3P binding synergy for glyphosate relative to the normal substrate, PEP, is consistent with numerous other studies (Sikorski et al., 1991) supporting Steinrücken and Amrhein's (1984) original hypothesis that glyphosate acts as a transition-state analogue for the transient PEP oxonium ion produced during catalysis.

Conclusions

For EPSP synthase, titration microcalorimetry offers several advantages over other binding techniques: (1) it is applicable to a broad range of complex affinities, (2) it does not require radiolabeled ligands, (3) it is not subjected to artifacts due to highly polar ligands interacting with membranes, and (4) it is not limited by an intrinsic physical parameter (e.g., fluorescence). The largest single limitation of this technique for the investigation of unique EPSPS complexes was the requirement for very large amounts of highly purified enzyme. Many of the experiments charaterizing weak complexes were carried out using enzyme concentration as high as 1 mM, near the limit of solubility in the buffer used, to provide a sufficient amount of heat upon titration for accurate characterization. In a typical weak complex experiment, approximately 8 mL of enzyme was used for three separate titrations. This requires an astonishing 365 mg of EPSPS *per determination* since the molecular weight is 45 650 (Duncan et al., 1984). Certainly this technique can be most usefully applied to enzymes that have been cloned and overexpressed in an appropriate organism to provide sufficient enzyme sample for study.

Nevertheless, this study characterizing the interaction of PEP, glyphosate, and P_i with free enzyme and the EPSPS·S3P complex by microcalorimetry has yielded tremendous new insights into our growing knowledge of this enzyme. As a result of this study, we have provided the first direct demonstration for an EPSPS·S3P·P_i ternary complex, elucidated an unexpectedly tight interaction between PEP and free enzyme, and provided additional experimental evidence to support glyphosate as a transition-state analogue. These results have had important implications for interpreting the overall kinetic reaction mechanism and have stimulated a variety of ongoing new studies for this important enzyme (Gruys et al., 1992).

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Substrate Synergism and the Steady-State Kinetic Reaction Mechanism for EPSP Synthase from *Escherichia coli*

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ABSTRACT: Previous studies of *Escherichia coli* 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) have suggested that the kinetic reaction mechanism for this enzyme in the forward direction is equilibrium ordered with shikimate 3-phosphate (S3P) binding first followed by phosphoenolpyruvate (PEP). Recent results from this laboratory, however, measuring direct binding of PEP and PEP analogues to free EPSPS suggest more random character to the enzyme. Steady-state kinetic and spectroscopic studies presented here indicate that *E. coli* EPSPS does indeed follow a random kinetic mechanism. Initial velocity studies with S3P and PEP show competitive substrate inhibition by PEP added to a normal intersecting pattern. Substrate inhibition is proposed to occur by competitive binding of PEP at the S3P site [$K_{i(PEP)}$ = 6–8 mM]. To test for a productive EPSPS·PEP binary complex, the reaction order of EPSPS was evaluated with shikimic acid and PEP as substrates. The mechanism for this reaction is equilibrium ordered with PEP binding first giving a K_{ia} value for PEP in agreement with the independently measured K_d of 0.39 mM (shikimate K_m = 25 mM). Results from this study also show that the 3-phosphate moiety of S3P offers 8.7 kcal/mol in binding energy versus a hydroxyl in this position. Over 60% of this binding energy is expressed in binding of substrate to enzyme rather than toward increasing k_{cat}. Glyphosate inhibition of shikimate turnover was poor with approximately 8×10^4 loss in binding capacity compared to the normal reaction, consistent with the independently measured K_d of 12 mM for the EPSPS·glyphosate binary complex. The EPSPS·glyphosate complex induces shikimate binding, however, by a factor of 7 greater than EPSPS·PEP. Carboxyallenyl phosphate and (*Z*)-3-fluoro-PEP were found to be strong inhibitors of the enzyme that have surprising affinity for the S3P binding domain in addition to the PEP site as measured both kinetically and by direct observation with ^{31}P NMR. The collective data indicate that the true kinetic mechanism for EPSPS in the forward direction is random with synergistic binding occurring between substrates and inhibitors. The synergism explains how the mechanism can be random with S3P and PEP, but yet equilibrium ordered with PEP binding first for shikimate turnover. Synergism also accounts for how glyphosate can be a strong inhibitor of the normal reaction, but poor versus shikimate turnover.

The enzyme 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS,[1] EC 2.5.1.19) catalyzes the unique transfer of a carboxyvinyl moiety from phosphoenolpyruvate (PEP) to shikimate 3-phosphate (S3P), forming EPSP (5-enolpyruvoylshikimate 3-phosphate) as shown in Scheme I [for reviews refer to Sikorski et al. (1991) and Anderson and Johnson (1990a)]. EPSPS plays an important role in the synthesis of essential aromatic amino acids and is the biological target for glyphosate, the active ingredient in Roundup herbicide (Amrhein et al., 1980; Franz, 1985). Data from this

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[1] EPSPS, 5-enolpyruvoylshikimate-3-phosphate synthase; S3P, shikimate 3-phosphate; PEP, phosphoenolpyruvate; EPSP, 5-enolpyruvoylshikimate 3-phosphate; EPS, 5-enolpyruvoylshikimate; 5-deoxy-S3P, 5-deoxyshikimate 3-phosphate; glyphosate, *N*-(phosphonomethyl)glycine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; P_i, inorganic phosphate; EPSP reaction, S3P and PEP turnover to produce EPSP; EPS reaction, shikimate and PEP turnover to produce EPS.

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