Kinetic and Structural Analysis of Enzyme Intermediates: Lessons from EPSP Synthase

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Contents

I. Introduction

The identification of intermediates during catalysis provides positive "proof" for a particular reaction pathway and, accordingly, many attempts to solve an enzymatic mechanism have centered on efforts to isolate or provide evidence for a given intermediate. However, the identification of an intermediate is only one part of a larger goal to establish a reaction sequence and to provide a complete kinetic and thermodynamic description of the catalytic pathway. Moreover, in the absence of a complete kinetic characterization, one cannot prove that a new species observed spectroscopically or isolated and identified chemically corresponds to a true reaction intermediate. In this review, we will examine the criteria used to establish a reaction pathway and to identify intermediates with a focus on the lessons learned in studying the EPSP synthase reaction. The approaches that led to the successful identification of the tetrahedral intermediate in the EPSP synthase reaction pathway will be chronicled, with an emphasis on the rationale for experimental design based upon a logical progression building upon the growing knowledge of the kinetics of the enzymatic reaction.

A number of intermediates in enzymatic reactions have been reported, and the evidence for these is supported in a variety of ways. Enzyme intermediates can be subdivided into three categories: (1) covalent enzyme–substrate adducts formed by reaction of an active-site nucleophile, (2) covalent adducts formed by reaction of the substrate with an enzyme cofactor, and (3) intermediates formed by the reaction of one or two substrates at the enzyme active site which then decompose to form products. Perhaps a fourth class of enzyme intermediates are those that represent distinct conformational states of the enzyme and are detected by rate-limiting transitions of the enzyme–substrate or enzyme–product complex. Such conformational transitions are increasingly common.[1-4] Although these "kinetic" intermediates are important to understanding the catalytic cycle, the analysis used to detect them will not be discussed further in this review, other than to state that the complete kinetic characterization of an enzyme reaction pathway will define the kinetically significant states and will often reveal conformational intermediates.

Most of the published evidence for intermediates involves covalent enzyme intermediates formed upon reaction of an enzyme nucleophile with the substrate. Examples for these include the acyl enzyme formed during peptide hydrolysis by serine proteases,[5,6] and the phosphoryl enzyme formed during hydrolysis of phosphate esters by alkaline phosphatase[7,8] or acid phosphatase.[9] The initial evidence for the serine protease acyl enzyme intermediate was based, in large part, upon the observation of a pre-steady-state burst of product formation and accumulation of the intermediate using synthetic substrates. These substrates were chosen because they reacted rapidly to form a covalent enzyme–substrate adduct, which then was more slowly hydrolyzed, thus leading to accumulation of the intermediate and allowing its identification. Synthetic substrates with different leaving groups gave the same rate of turnover, supporting the common acyl enzyme intermediate whose hydrolysis was rate limiting.[5,6] Although the data in support of an acyl enzyme intermediate are certainly unequivocal, an acyl enzyme has not been observed with natural substrates because its formation is rate limiting and it does not accumulate.[5,6] Indeed, it has been argued that enzymes evolve to select

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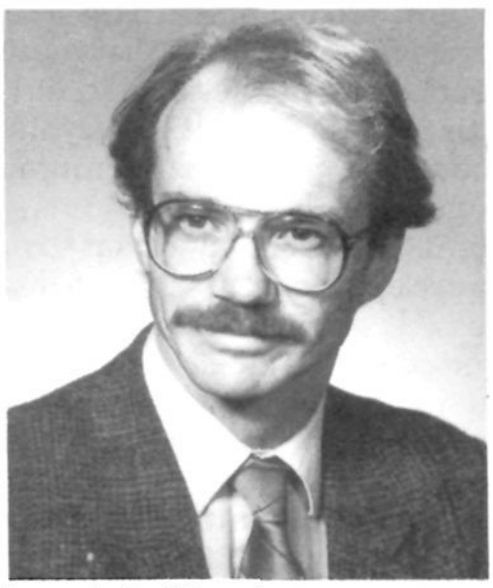


Kenneth A. Johnson is Paul Berg Professor of Biochemistry at The Pennsylvania State University at University Park, PA, and is the 1989 recipient of the Pfizer Award in Enzyme Chemistry. After earning a B.S. in Chemistry at the University of Iowa (1971), he received his Ph.D. in Molecular Biology at the University of Wisconsin for work on the pathway of microtubule assembly with Gary Borisy (1975). He obtained postdoctoral training with Edwin W. Taylor at the University of Chicago, where he learned transient kinetic methods. Since joining the faculty at Penn State in 1979, Dr. Johnson has worked toward the refinement and application of transient kinetic methods using stopped-flow and chemical-quench flow instruments constructed in his laboratory. This work has led to the solution of several enzyme pathways, including those of the dynein ATPase, T7 DNA polymerase, DNA polymerase I, and dihydrofolate reductase, the latter two in collaboration with Steve Benkovic.

against the accumulation of enzyme-bound intermediates in their drive to improve the efficiency of catalysis.[10,11]

There is good kinetic evidence for covalent intermediates involving reactive cofactors. For example, the reactions catalyzed by pyridoxal phosphate involve a number of intermediate structures with unique spectral properties which have allowed their identification and the measurement of their time dependence of formation and decay.[12-15] In addition, major advances have been made in our understanding of the reactions of other enzyme cofactors including thiamin pyrophosphate.[16,17]

In this review, we will focus on studies in which an enzyme intermediate is formed by the reaction of one or two substrates. It is in these instances where the identification of the intermediate is central to proof of the reaction mechanism. In mechanisms that do not proceed via a covalent enzyme intermediate or by a reaction involving an enzyme cofactor, the number of alternate mechanisms can be quite large and difficult to resolve. One classical approach to solving such enzyme mechanisms is based upon the synthesis and evaluation of analogues of putative enzyme intermediates, the rationale being that if an analogue binds tightly, it must mimic a true intermediate. Alternatively, in favorable instances, isotope tracer methods have identified those bonds which are broken in the substrate, thereby limiting the number of possible mechanisms.

There are only a few cases in which direct evidence has been obtained for an intermediate formed upon reaction of one or two substrates. Perhaps the most abundant bisubstrate intermediate is formed by reaction of CO_2 with ribulose 1,5-bisphosphate, catalyzed by the enzyme ribulose bisphosphate carboxylase. This intermediate was originally proposed by Calvin[18] and was recently isolated in the keto form by Pierce and co-workers.[19,20] Other cases involve studies on synthetases, where reaction with ATP is thought to produce a reactive intermediate necessary to drive the synthetic reaction. For example, the reaction catalyzed by N^{10}-formyltetrahydrofolate synthetase is thought to proceed by the reaction of formate with ATP to make formyl phosphate, which then reacts with tetrahydrofolate (THF) to form N^{10}-formyltetrahydrofolate.[21,22] In an analogous reaction catalyzed by glutamine synthetase, ATP and glutamate are thought to react to form γ-glutamyl phosphate, which then reacts with ammonia to form glutamine.[23-25] In both of these cases, all three substrates must be present for reaction to occur at optimal rates and the intermediate cannot be observed under these conditions, presumably because of its rapid rate of reaction. Nonetheless, good evidence for the existence of these intermediates has been derived from a number of studies including isotope-exchange methods and by the evaluation of novel substrate analogues and inhibitors. For example, methionine sulfoximine, a tight binding inhibitor of glutamine synthetase, binds to the enzyme active site and is phosphorylated in the presence of ATP to form a close structural homologue of γ-glutamyl phosphate.[26] These data provide support for the existence of γ-glutamyl phosphate as a reaction intermediate, although it is unstable and cannot be isolated. On the other hand, formyl phosphate is stable and can be synthesized; it reacts when added back to formyl-THF synthetase in the presence of ADP and THF, although at a rate too slow to account for turnover.[21] After preincubation of enzyme with ADP and formyl phosphate, the addition of THF results in a rapid burst of synthesis which is faster than turnover with the normal substrates. In addition, formyl phosphate can be formed from ATP

and formate in the absence of THF, but at a rate 100-fold slower than turnover in the presence of THF.[22]

The reaction catalyzed by the enzyme triosephosphate isomerase proceeds via an enediol intermediate.[27-29] There is substantial kinetic evidence for the existence of the intermediate,[30] but it cannot be isolated because it rapidly decomposes in solution to yield methylglyoxal and phosphate. Several intermediates have been postulated in essential amino acid biosynthesis on the basis of the ability of the enzymes to utilize synthetically prepared intermediates at rates comparable to product formation using natural substrates.[31-34] These data support the conclusion that the postulated intermediate reacts fast enough to account for the normal enzymatic reaction, but it remains to be established that the intermediate is formed at the active site at a rate fast enough to account for turnover.

Perhaps the most definitive evidence for an intermediate has been derived from studies on aminoacyl-tRNA synthetases.[35] In those enzymes that have been investigated most thoroughly, the amino acid reacts with ATP to form aminoacyl-AMP, which then reacts with tRNA to form the aminoacyl-tRNA. Because the aminoacyl-AMP is formed in the absence of the third substrate and is held tightly at the enzyme site until tRNA binds, the existence of the intermediate was relatively easy to establish.[36] However, direct kinetic analysis was required to establish the kinetic competence of the intermediate, by showing that it was formed and reacted at a rate sufficient to account for the net synthetic reaction in the presence of tRNA.[3]

A. Criteria for Identification of Enzyme Intermediates

In studies on EPSP synthase, the isolation of the enzyme intermediate provided definitive proof for the mechanism and afforded unique opportunities to examine the reactions of the enzyme to catalyze both the formation and decay of the intermediate and to characterize the binding of the intermediate to the enzyme. The studies on EPSP synthase serve as a focal point for discussions on the methods and rationale for experiments designed to identify enzyme intermediates because they point to the importance of the interplay of kinetic and structural information to establish a reaction mechanism. Methods of analysis developed in solving the EPSP synthase mechanism may prove useful when applied to other enzyme systems. We will describe the experimental approaches in terms of how each contributed to discovering the intermediate and proving the reaction pathway.

The criteria to establish an enzymatic reaction pathway with a postulated intermediate can be defined by addressing the following questions:

1. Kinetic Competence. Are the time dependence of formation and decay of the intermediate *at the active site* fast enough to account for rates of conversion of substrate to product in both forward and reverse reactions? Steady-state kinetic analysis to test kinetic competence of an intermediate, which is also a function of the binding of the intermediate to the enzyme does *not* serve to define kinetic competence.

2. Structure Proof. Can the intermediate be isolated and its structure determined directly? If the intermediate is unstable, can the analysis of its breakdown products support the postulated structure?

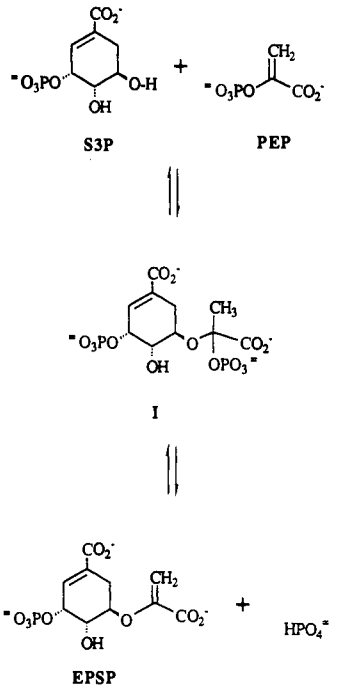
3. Detection of Enzyme-Bound Intermediate. Can the intermediate be observed bound to the enzyme by spectroscopic methods and quantitated by rapid quenching methods?

4. Thermodynamic Analysis of Pathway. Are all the data quantitatively consistent with a single reaction pathway such that all kinetically and thermodynamically significant enzyme states have been described?

5. Chemical Rationale. Is the proposal of the reaction intermediate based upon chemical precedent, reasonable thermodynamics, and maintenance of microscopic reversibility?

Recent studies have demonstrated a tetrahedral intermediate in the EPSP synthase reaction pathway shown in Scheme I which satisfies all of the above criteria. Thus, EPSP synthase provides a prime example illustrating the approaches used to establish an enzymatic reaction pathway. Moreover, the analysis follows a logical progression from the initial steady-state and equilibrium binding studies, to transient-state kinetic studies demonstrating the appearance of the intermediate, which led to its isolation and identification and finally to its observation bound to the enzyme under equilibrium conditions. In this review, we will explain the rationale for each approach and describe alternative attempts that were unsuccessful.

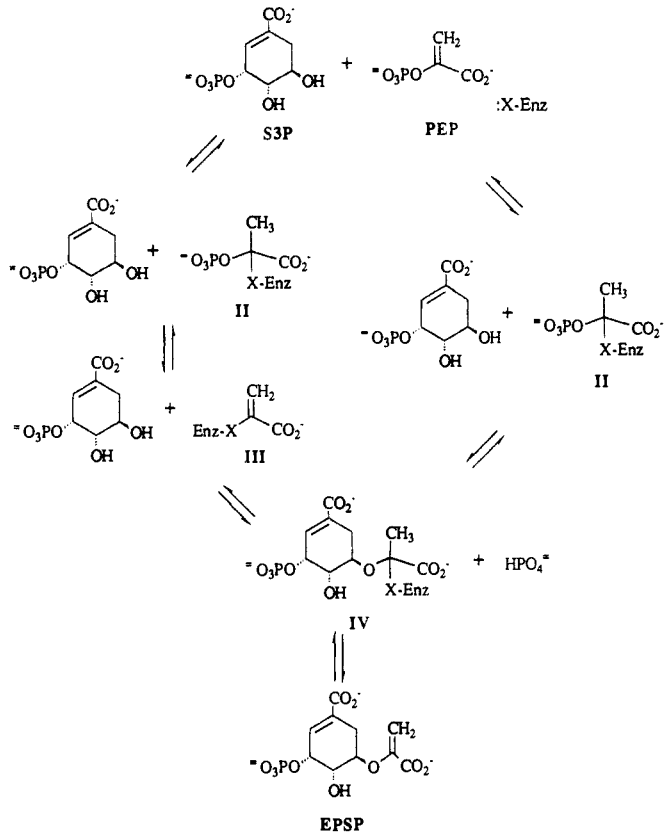
SCHEME I. EPSP Synthase Reaction Pathway



B. Previous Mechanistic Studies on EPSP Synthase

EPSP synthase is an enzyme in the shikimic acid pathway found in plants and bacteria and the target of the commercially important herbicide glyphosate.[37-39] The unusual biochemical reaction catalyzed by the enzyme is shown in Scheme I, where an enolpyruvoyl group is transferred from phosphoenol pyruvate (PEP) to the 5-OH of shikimate 3-phosphate (S3P) to form the products, enolpyruvoylshikimate 3-phosphate (EPSP) and inorganic phosphate (P_i). Only one other enzyme is known to catalyze the transfer of an enolpyruvoyl

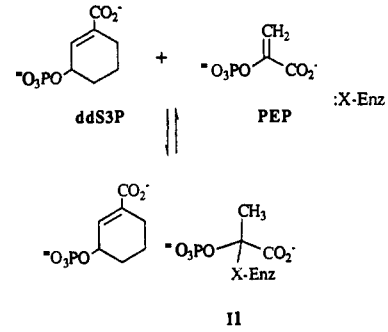
SCHEME II



moiety from phosphoenol pyruvate, UDP-*N*-acetylglucosamine enolpyruvoyl transferase, which catalyzes the first committed step in biosynthesis of bacterial cell wall peptidoglycan by transferring the enolpyruvoyl moiety of PEP to the oxygen attached to C-3 of the glucosamine moiety of UDP-*N*-acetylglucosamine.[40] Studies on this enzyme have suggested a covalent enzyme–enolpyruvoyl intermediate on the basis of the observation of traces of radioactivity associated with the protein following incubation with radiolabeled PEP. These results, although far from conclusive, greatly influenced the progress of research on EPSP synthase and led to numerous attempts to look for a similar covalent intermediate.[41,42]

The pioneering work on EPSP synthase, from Sprinson's laboratory,[43] provided two important mechanistic details. First, during the reaction the methylene protons of PEP exchanged with solvent, indicating the formation of a transient methyl group with a tetrahedral center at C-2 of PEP. Second, the reaction was shown to proceed with C–O bond cleavage in the elimination of phosphate. Accordingly, Sprinson provided the most simple explanation of these observations by proposing the addition–elimination mechanism as shown in Scheme I. A decade later, work from Knowles's laboratory confirmed the observation of proton exchange and extended these results to show a primary isotope effect on the protonation and deprotonation steps.[44] Floss and co-workers also demonstrated a similar isotope effect but suggested that the results supported a covalent enzyme intermediate.[45] However, in reality, each of these results does not distinguish between the addition–elimination mechanism and a more complex pathway involving the addition of PEP to an enzyme nucleophile such as serine according to one of the reaction pathways shown in Scheme II.

SCHEME III



The first direct support for the involvement of an enzyme nucleophile came from Abeles's laboratory.[41] In the presence of dideoxy-S3P, an analogue lacking the hydroxyl groups at the 4- and 5-positions of the shikimate ring, the enzyme catalyzed the exchange of the methylene protons of PEP. Because the shikimate 5-OH group required to react with C-2 of PEP to form the tetrahedral center was missing, it was reasoned that an enzyme nucleophile must react with the PEP to catalyze the proton exchange as illustrated in Scheme III.

At this point candidate pathways existed, one involving a simple addition–elimination mechanism (Scheme I) and another involving some covalent enzyme species, with numerous possible variations upon the theme as shown in Scheme II. We, like many others, attempted to trap a covalent enzyme–enolpyruvoyl intermediate by incubating enzyme with [^{14}C]PEP, quenching, and then looking for counts associated with the protein. However, these experiments are not terribly robust, and the failure to observe radioactivity associated with the protein is inconclusive. The largest limitation of this approach is due to the difficulty of locating an intermediate in the undefined time–concentration space. What concentrations of S3P and PEP are required to saturate enzyme with intermediate, and what time of incubation is optimal? Should the reaction be run in the forward or reverse directions for the intermediate to accumulate? To answer these questions, direct analysis of the kinetics of the reactions occurring *at the enzyme active site* is required.

Steady-state kinetic analysis does not provide information to define the reactions occurring at enzyme active sites. To establish an active-site enzymatic reaction pathway, the enzyme must be examined as a stoichiometric reactant, not just as a trace catalyst. In general, three important things are learned from analysis of enzyme kinetics in the steady state: (1) The possible order of substrate binding and product release can be determined, which allows one to infer which substrates are at the active site during catalysis. However, the kinetically preferred pathway of binding cannot be established, and the events governing the conversion of substrate to product at the active site collapse into a single, net rate constant. (2) The maximum velocity defines the net rate of conversion of substrate to product and therefore sets a lower limit on any given step in the pathway as greater than or equal to k_{cat}. (3) The substrate concentration dependence of the rate defines the *apparent* second-order rate constant for substrate binding, by the term k_{cat}/K_m, which sets a lower limit on the true second-order rate constant for substrate binding ($k_{on} \geq k_{cat}/K_m$). The Michaelis

constant can be viewed as the ratio of k_{cat} and the apparent second-order rate constant for substrate binding. As such, it is more readily recognized that the magnitude of K_m is largely uninterpretable in the absence of more definitive mechanistic information. In reality, the K_m can be greater than, less than, or equal to the substrate equilibrium dissociation constant, K_d.

Although steady-state kinetic analysis does not provide mechanistic information required to distinguish alternate active-site mechanisms, it does provide crucial information required to design more definitive experiments. For example, the steady-state kinetic analysis of EPSP synthase provided three important pieces of information: (1) The pathway of substrate binding appeared to be ordered with S3P binding first and PEP second;[46] (2) the K_m values for both substrates were in the micromolar range;[47] and (3) the k_{cat} values were 60 s^{-1} for the forward reaction and 10 s^{-1} in the reverse reaction.[46] These observations influenced the progress of subsequent work in several ways: (1) The ordered-sequential binding indicated that both substrates must be present for reaction to occur; for example, a ping-pong mechanism with the transient formation of an enzyme–enolpyruvoyl intermediate after reaction with PEP can be eliminated from consideration. In addition, the suggestion that both reactants must be present influences the design of direct attempts to establish the reaction pathway. (2) The low K_m values allow one to perform rapid chemical quench flow experiments in the pre-steady state and under single-turnover conditions to examine directly the reactions at the active site of the enzyme; these experiments can best be performed when the concentration of enzyme is comparable to the K_m values for the substrates. (3) The moderate turnover rate of 60 s^{-1} ($t_{1/2}$ = 11.6 ms) is well within the time domain of rapid mixing methods and sets a limit for the slowest step in the pathway. It should be noted that enzymes with K_m values in the millimolar range and k_{cat} values in excess of 500 s^{-1} are not readily amenable to the rapid chemical quench flow methods.

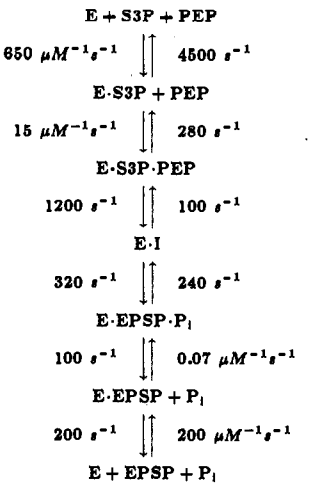
II. Complete Kinetic Description of the EPSP Synthase Reaction

A complete kinetic and thermodynamic analysis provides rigorous proof for the mechanism and establishes the thermodynamic basis for the enzyme-catalyzed reaction. The goals of a complete kinetic analysis are to estimate the elementary rate constants for each step in the pathway, thereby defining the free energy path taken in the conversion of substrate to product in relationship to the pathway taken for the uncatalyzed reaction. In addition, the role of the enzyme in selective stabilization or destabilization of the intermediate can be addressed by analysis of thermodynamics of the reaction pathway, as well be discussed under section IV of this review.

Such complete information is available for only a handful of enzymes,[2,4,30,48] and the methods that have been employed to solve each mechanism have depended upon the nature of the enzyme-catalyzed reaction. Therefore, it is instructive to examine the analysis that led to solution of the EPSP synthase mechanism.

The availability of computer programs to analyze kinetic data by numerical integration of the complex rate equations has greatly influenced the way in which

SCHEME IV. EPSP Synthase Kinetic Pathway



experiments are conducted and interpreted. The 12 rate constants derived for the EPSP synthase reaction are shown in Scheme IV. To solve the EPSP mechanism, all the rate constants for the pathway were obtained by fitting a large number of separate experiments to one complete model by computer simulation, using our modification of the program KINSIM, provided to us by Carl Frieden.[48,49] Previously, the standard protocol has been to extract observed rates and amplitudes from kinetic data and then to interpret the observed, *apparent* constants in terms of the combinations of intrinsic rate constants that they represent. There are obvious limitations to that approach, largely due to the loss of information and errors in extracting kinetic constants from data. The study of EPSP synthase sets a new standard in which the data are fitted directly to the model by computer simulation. According to this approach, every experiment is quantitatively interpreted by a single chemical mechanism, with no simplifying assumptions. This approach has recently been successful in studies to establish the pathway of DNA polymerization with a single, thermodynamically complete description of the elementary steps in DNA replication.[2] Use of the program KINSIM was also essential in solution of the reaction pathway of dihydrofolate reductase.[4]

To achieve a complete description of an enzymatic reaction pathway, experiments must be designed that attempt to measure each step of a reaction. The difficulty in achieving this goal arises from the evolution of catalytic efficiency, which has tended to result in enzymatic pathways where the rates of all reactions are comparable.[30] Certainly this is true in the case of EPSP synthase, and it represents another reason for the need to fit each experiment to the complete model, with no simplifying assumptions. The challenge then is to perform experiments that will provide rate and equilibrium information most strongly dependent upon one reaction step and only secondarily dependent upon others in the sequence.

It is most logical to begin at the two ends of the reaction sequence and work our way inward. This is the sequence in which the EPSP synthase experiments were performed and can best be understood. Moreover, each experiment builds upon its predecessors in working toward the center of the pathway. Accordingly, we begin with equilibrium binding measurements for the

substrate S3P and the product EPSP.

A. Equilibrium Binding Studies

Accurate estimates of the magnitude of the binding constants for substrates and products are important for several reasons. First, they influence the design and the quantitative interpretation of substrate trapping and single-turnover experiments described below. Second, to complete the thermodynamic description of the reaction pathway, it is necessary to know the free energies for each step of the reaction to ensure that they add up to the net free energy change for the overall reaction in solution. Of course, equilibrium binding measurements can only be performed for substrates that bind but do not react, namely, the first substrate to bind in an ordered, multiple-substrate reaction. Alternatively, substrate dissociation constants have been estimated as the ratio of dissociation and binding rates from kinetic measurement.[4,50]

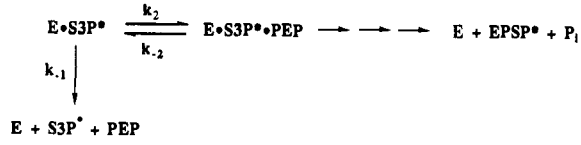
Equilibrium binding measurements are often the most difficult to perform accurately, because of the potentially large systematic errors and the narrow dynamic range of the methods. It is difficult to measure dissociation constants less the 0.1 μM because it is imperative that the measurements be performed with enzyme concentrations no more than 10-fold greater than the K_d. At ratios of $[E_0]/K_d > 10$, an active-site titration can accurately define the concentration of enzyme sites, but attempts to extract binding constants lead to large errors. On the other extreme, one is often limited by solubility of the substrate or adverse ionic effects at concentrations of substrates exceeding 10 mM. Within this range, accurate measurements can be obtained if care is taken to ensure the attainment of equilibrium and with proper fitting of data to the quadratic equation allowing for variation in active enzyme site concentrations and in the end points of the titration as unknown variables.[51,52]

Measurements of the equilibrium constants for S3P and EPSP binding to EPSP synthase were obtained by fluorescence titrations, taking advantage of changes in protein fluorescence upon the binding of EPSP or S3P and glyphosate. From these measurements K_d values of 7 and 1 μM were obtained for S3P and EPSP, respectively.[51]

B. Stopped-Flow Kinetic Measurements

As an alternative to measuring the dissociation constant directly, it is often possible to measure binding and dissociation rates and then estimate the K_d from their ratio. For example, stopped-flow measurements of protein fluorescence changes have provided convenient methods to measure the rates of substrate binding and reaction for several enzymes.[1,4,25] Here the major limitation is that the observed rate is the sum of the binding and dissociation rates, and for fast off-rates, it may be impossible to observe the reaction. For example, the dissociation rate of EPSP is 200 s^{-1}, and therefore one expects a rate of 1000 s^{-1} at a concentration of EPSP at only 4 times the K_d ($k_{obs} = k_{on}$[EPSP] + k_{off}). Accordingly, all attempts to measure the kinetics of EPSP binding by stopped-flow methods have failed, and only a lower limit on the rate of S3P binding could be obtained. Nonetheless, estimates of the S3P and EPSP binding and dissociation rates could be obtained by substrate trapping methods.

SCHEME V



C. Substrate Trapping Experiments

Substrate trapping experiments, which were originally pioneered by Rose,[53] provide a method to examine the kinetic partitioning between dissociation of the substrate from the enzyme and the forward reaction dependent upon the binding of the second substrate. For example, EPSP synthase was incubated with [^{14}C]S3P under conditions where a large fraction of the S3P would be bound to the enzyme, according to the known K_d for S3P. The reaction was then initiated by mixing with PEP and an excess of unlabeled S3P. The experiment measures the kinetic partitioning between dissociation of the [^{14}C]S3P from the enzyme and the reaction of PEP with E·[^{14}C]S3P to drive the reaction forward as shown in Scheme V. The fraction of [^{14}C]S3P that is converted to EPSP in a single enzyme turnover defines the relative magnitudes of the rate constants governing dissociation of S3P and the binding of PEP. According to this analysis, which is explained in depth in the original publication,[48] the rate of S3P dissociation was estimated to be 4500 s^{-1}, indicative of a truly rapid equilibrium binding of S3P with the enzyme.

One issue has not yet been resolved. As the concentration of PEP increased, the fraction of S3P that was trapped by the PEP tended toward 70%, not 100%. The complete analysis of all possible alternative explanations of this result, including random dissociation, ended with the conclusion that 30% of the S3P was bound to the enzyme in a "nonproductive" complex,[48] requiring dissociation of S3P into solution prior to the productive binding of the PEP. One possible explanation of this result is that an active-site base required to remove the proton from the 5-OH of S3P may be in the wrong ionic state for catalysis to occur in the nonproductive complex and the S3P must dissociate to make the enzyme residue accessible to solvent. This explanation may provide some rationale for the maintenance of a rapid equilibrium binding of S3P in spite of the apparent evolutionary pressures that might be imagined to reduce the dissociation rate to improve catalytic efficiency. Further work will be required to establish the structural basis for nonproductive binding of S3P by EPSP synthase.

D. Overall and Internal Chemical Equilibria

Measurements of the overall chemical equilibrium constant for the reaction in solution and the internal equilibrium for the reaction at the active site of the enzyme are important because they define the free energy changes governing the conversion of substrates to products. Measurement of the overall equilibrium constant for EPSP synthase was relatively easy to achieve by reacting a trace of enzyme with EPSP labeled both in the shikimate ring and in the enolpyruvoyl moiety and with various concentrations of

unlabeled phosphate. Following incubation to attain equilibrium (3 h), the reaction mixture was resolved by HPLC and the equilibrium constant calculated directly by the ratio $K_{overall}$ = [EPSP][P_i]/[S3P][PEP] = 180.

The internal equilibrium involving the conversion of substrates to products at the enzyme active site was also measured directly. From knowledge of the equilibrium constant for the binding of substrates and products to the enzyme, conditions could be designed under which all of the species containing the shikimate ring would be bound to the enzyme. A high concentration of PEP served to drive the reaction forward while a high concentration of phosphate served to drive the reaction backward:

$$E\cdot S3P + PEP \rightleftharpoons \{E\cdot S3P\cdot PEP \rightleftharpoons E\cdot I \rightleftharpoons E\cdot EPSP\cdot P_i\} \rightleftharpoons E\cdot EPSP + P_i$$

With radiolabel in the shikimate ring, the internal equilibrium could be quantitated directly by measuring the distribution of label between S3P, I, and EPSP by HPLC following rapid quench with base or triethylamine.[48,54] In initial experiments based upon quenching with acid, the intermediate decomposed to form S3P and pyruvate; therefore, the equilibria could not be resolved into two steps.[48] However, in subsequent experiments as described below,[54-56] conditions for stabilization of the intermediate at high pH allowed direct quantitation of both equilibria, giving 4% S3P, 33% I, and 61% EPSP, thus helping to define the equilibrium constants describing the reactions at the active site according to Scheme IV.

In a recent paper, this measurement of the internal equilibrium was mistakenly described as a "steady-state quench".[57] A steady-state quench would involve the attempt to resolve an intermediate or enzyme-bound product from the rapidly accumulating product in solution. This type of experiment is a method of last resort to be applied only when the reaction is so irreversible that an internal equilibrium cannot be achieved. There is no question that the internal equilibrium measured in the case of EPSP synthase represents a true equilibrium between substrates, intermediate, and products at the active site. The magnitudes of the equilibrium constants for both steps are supported by direct measurement of the substrate → intermediate → product conversion in each direction (see below) and by the measurement of the equilibrium constant for the remaining steps in the pathway, such that the product $K_1K_2K_3K_4K_5K_6 = K_{overall}$. Moreover, computer simulation of the kinetics of approach to the equilibrium was used to check the validity of the conditions used to make the measurements in terms of consistency with all known kinetic parameters.[48] In the present case, equilibrium is reached within 50 ms, but for convenience a reaction time of 5 s was chosen because no side reactions occurred over this longer time interval.

E. Single-Turnover Experiments

The most definitive experiments to examine the reactions at the active site of the enzyme involve following the conversion of substrate to product in a single enzyme turnover with enzyme in excess over the limiting substrate containing the radioactive label. These experiments allow the direct observation of events occurring at the active enzyme sites, and because the reaction proceeds with 100% conversion of substrate to product, the sensitivity for detection of any enzyme-bound intermediates is optimal. To see the buildup of any transient intermediates, the most important kinetic requirement of these experiments is that the enzyme concentration is high enough to provide a rate of substrate binding faster than turnover. Because enzyme is in excess, the rate of substrate binding is governed by the pseudo-first-order rate constant defined by the product k_{on}[enzyme]. Thus, proper design of the experiment depends upon an estimate of the rate of substrate binding and a knowledge of k_{cat}. Rapid chemical quench flow methods allow examination of the reaction on a time scale of milliseconds usually required for a single turnover.[58]

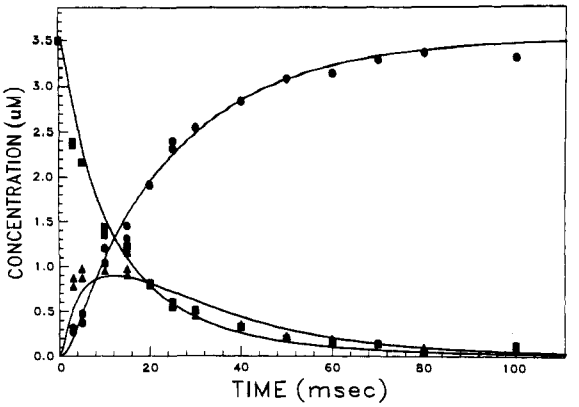


Figure 1. Single turnover in the forward reaction. A solution of S3P and enzyme was mixed with a solution of [^{14}C]PEP to initiate the reaction. The final concentrations were 100 μM S3P, 10 μM enzyme, and 3.5 μM [^{14}C]PEP. The formation and disappearance of pyruvate (▲), PEP (■), and EPSP (●) were monitored. The curves were calculated by the numerical integration using the rate constants summarized in Scheme IV. (Reprinted with permission from ref 48. Copyright 1988 American Chemical Society.)

The first experiment was designed to investigate a single turnover of the enzyme-catalyzed reaction in the forward direction. A high concentration of enzyme saturated with unlabeled S3P was mixed with a limiting concentration of radiolabeled PEP. The results of this experiment are plotted in Figure 1, showing the time dependence of the disappearance of radiolabeled PEP (■), the appearance of radiolabeled EPSP (●), and the transient rise and decay of an intermediate (▲). The intermediate, present at 5 ms, reached a peak in concentration at 15 ms and was completely consumed by 50 ms. The curves shown were simulated by numerical integration using the KINSIM program[49] using the rate constants shown in Scheme IV. As rigorously defined by the simulation, the formation and decay of intermediate exactly paralleled the disappearance of substrate and the appearance of product within a single enzyme turnover, thus satisfying the criteria for a reaction intermediate.

This experiment provided the first evidence for an intermediate in the EPSP synthase reaction. Although the time dependence of the reactions could be fit by a single set of rate constants governing the reactions at the active site, the fit was not unique in that several possible sets of rate constants could account for the data in this one experiment. However, because the reaction was freely reversible, it was then possible to perform a single-turnover experiment in the reverse direction

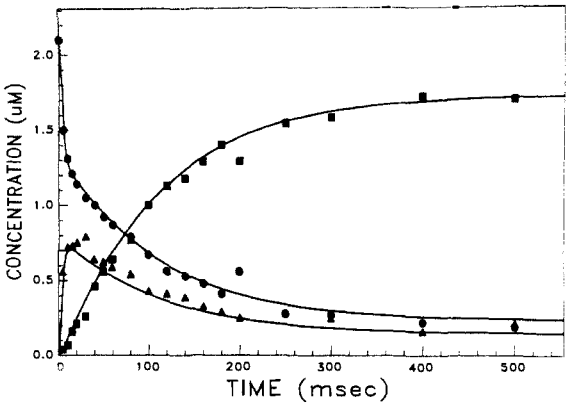


Figure 2. Single turnover in the reverse reaction. A solution of enzyme was mixed with a solution of [^{14}C]EPSP and phosphate. The final concentrations were 10 μM enzyme, 2.1 μM [^{14}C]EPSP, and 7.5 mM phosphate. The formation and disappearance of pyruvate (▲), EPSP (●), and PEP (■) were monitored. The curves were simulated by the numerical integration using the rate constants summarized in Scheme IV. Fitting the amplitudes of these curves required the additional constraint that phosphate binds to E·S3P complex with a dissociation constant of 0.67 mM. (Reprinted with permission from ref 48. Copyright 1988 American Chemical Society.)

and thereby further constrain the possible fits to the data.

Thus, the second experiment was designed to examine the conversion of EPSP to PEP and S3P in a single turnover. A high concentration of enzyme was mixed with a limiting amount of radiolabeled EPSP and a high concentration of phosphate, to drive the reaction in reverse. As shown in Figure 2, the kinetics are markedly biphasic. The intermediate (▲) is formed rapidly at the expense of EPSP (●) in a rapid phase peaking at 30 ms followed by a slower phase where the two species decay together in parallel as PEP (■) is formed. The kinetics can be easily understood in terms of the rate constants in Scheme IV. A rapid equilibrium at the enzyme active site is established between EPSP and intermediate at a rate of 240 s^{-1} + 320 s^{-1} = 560 s^{-1}. The breakdown of the intermediate in the reverse direction is then rate limiting at a rate of 100 s^{-1}. The calculated curves in Figure 2 again establish that the intermediate is formed and broken down at a rate which is consistent with substrate disappearance and product formation within a single enzyme turnover.

The combination of the two experiments describing single turnovers in the forward and reverse directions and definition of the internal equilibrium constants sufficiently constrain the fitting of the data to a single unique set of rate constants. This, in turn, provides evidence for a single kinetically significant intermediate along the reaction pathway. These data argue in favor of a simple addition–elimination mechanism and against a more complex reaction sequence involving multiple intermediates. More importantly, however, these experiments defined the conditions under which the intermediate could be formed at the active site of the enzyme, leading to its isolation to provide final proof of the reaction mechanism.

III. Isolation, Identification, and Characterization of the Intermediate

In the single-turnover experiments described above, the intermediate itself was not observed directly but

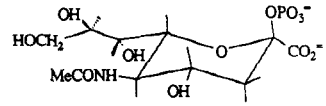
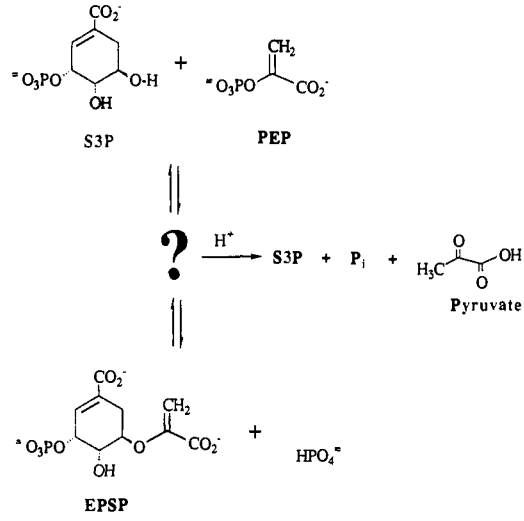


Figure 3. Neuraminic acid derivative containing functionality similar to that of the intermediate.

SCHEME VI



rather the breakdown products of the intermediate in the acid quench used to stop the reaction, S3P, pyruvate, and phosphate, as illustrated in Scheme VI. Therefore, evidence for the intermediate was somewhat indirect and was obtained only in those experiments where the radiolabel was contained in the enol portion of PEP or EPSP, leading to the formation of radiolabeled pyruvate. Because it is chemically unreasonable to suggest that pyruvate is actually the intermediate in the reaction, it was most reasonable to suggest that it formed as a breakdown product of an acid-labile intermediate under the acidic quench conditions. The question then remained as to the identity of the intermediate.

At this point, two properties of the intermediate were known. First, it breaks down to pyruvate under acidic conditions, and second, it must have a structure that accounts for the exchange of the methylene protons of PEP with solvent during formation and decay of the intermediate during catalytic turnover. Each of these properties points to a structure in which a methyl group attached to a tetrahedral center formed at C-2 of PEP is created. The only question then is the identity of the group attached to the tetrahedral center, either the shikimate ring or an enzyme nucleophile. The data by Anton et al. suggested that the group attached to the tetrahedral center is an active-site nucleophile such that the intermediate is covalently attached to the enzyme (Scheme III). Alternatively, the group could be the shikimate ring such that the intermediate is the simple addition product of S3P to a protonated carbonium ion form of PEP (Scheme I). In either case the chemical stability and possibility of isolation of such an intermediate were uncertain. Previous studies have characterized a neuraminic acid derivative with a similar tetrahedral center as shown in Figure 3, which had been reported to be moderately stable under mildly basic conditions.[59]

To isolate an intermediate with similar functional groups, it was thought to be necessary to denature rapidly the enzyme under mildly basic conditions. After an extensive search of quenching agents, it was found that neat triethylamine would rapidly denature the enzyme and provide a final pH of 12. According to the previously measured rate constants and substrate binding order, an internal equilibrium at the enzyme active site could be established between S3P, intermediate, and EPSP by adding a high concentration of enzyme to a limiting amount of S3P in the presence of PEP and P_i. A saturating concentration of PEP would drive the reaction forward, and a high concentration of phosphate would be required to push the reaction in the reverse to maintain an equilibrium between S3P, intermediate, and EPSP bound to the enzyme. The equilibrium distribution of these species was then measured by using S3P containing radiolabel in the shikimate ring. After the internal equilibrium mixture was quenched with neat triethylamine and after analysis by HPLC anion-exchange chromatography, three peaks were observed: S3P, EPSP, and a later eluting more highly charged compound. Since the radiolabel was contained in the shikimate ring, this result indicated that the third peak corresponded to the intermediate, I, shown in Scheme I.

The intermediate was also synthesized enzymatically with the radiolabel in the carboxyl group of PEP and the phosphate of PEP and in each case the same late-eluting radiolabeled peak was observed in the HPLC, confirming that the intermediate contained a shikimate ring, phosphate, and carboxylate moieties. Although the chemical evidence for the tetrahedral intermediate was convincing, more definitive structural proof was provided by NMR.

Enzymatic synthesis of the intermediate using PEP with carbon-13 in the C-2 position produced intermediate which was labeled with ^{13}C at the tetrahedral center. Because the yield of I is only 0.3 per enzyme site, this was a difficult enzymatic synthesis with an overall yield of 300 μg from 0.5 g of enzyme (a procedure not recommended on a daily basis!). Nonetheless, sufficient intermediate was purified to perform the NMR measurements. The ^{13}C spectrum showed a single resonance at 101.7 ppm, split into a doublet due to carbon–phosphorus coupling. In addition, the ^{31}P spectrum revealed a unique resonance for the phosphate attached to the tetrahedral center which was split into a doublet due to coupling with the adjacent carbon-13. The proton spectrum provided the definitive structural proof by the resonance observed at 1.7 ppm for the methyl group which was also coupled to the tetrahedral carbon.[55] These spectra provided final structural proof for the tetrahedral intermediate. Together with the kinetic properties, the isolation of the intermediate established the enzymatic pathway unequivocally.

A. Direct Observation of the Intermediate Bound to Enzyme

Identification of the intermediate and demonstration of its time dependence of formation and decay are sufficient to establish the mechanism involving the tetrahedral intermediate. Moreover, these data provided needed information to allow the interpretation of NMR spectra designed to examine products bound

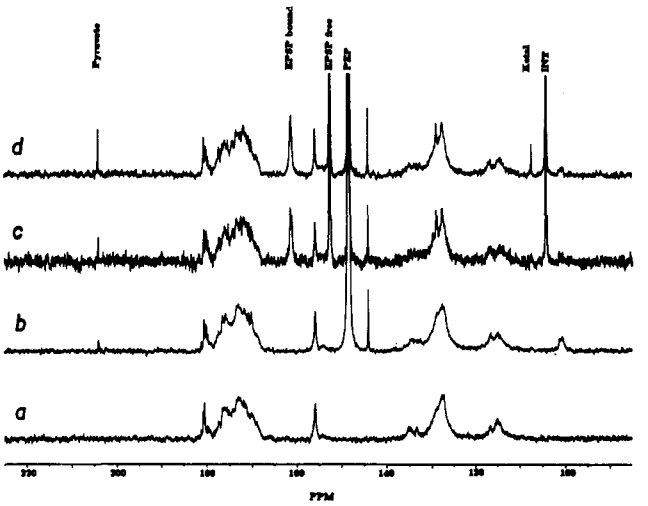


Figure 4. ^{13}C NMR spectrum of the tetrahedral intermediate bound to the enzyme active site. (a) Downfield region of proton-decoupled natural abundance ^{13}C NMR spectrum of EPSP synthase (1.3 mM) in 50 mM HEPES and 50 mM KCl in D_2O containing 10% glycerol at pH 7.0. (b) Downfield region of the proton-decoupled ^{13}C NMR spectrum of EPSP synthase (1.3 mM) plus [2-^{13}C]PEP (6 mM) and potassium phosphate (15 mM). Two new resonances were observed upon the addition of PEP. A large peak appearing at 148 ppm was assigned to the carbon 2 position of PEP and a small resonance at 144 ppm which was shown to arise from a minor contaminant present in the commercial preparation of [2-^{13}C]PEP. In addition, a broad resonance of low intensity was observed at 101 ppm, which could be due to natural abundance at C-3 of PEP. (c, d) Downfield region of the proton-decoupled ^{13}C NMR spectrum of EPSP synthase (1.3 mM), with [2-^{13}C]PEP (6 mM) and S3P (3 mM). Spectrum c was obtained within 1 h after the addition of S3P (4000 scans), while spectrum d was obtained after 4.5 h (17 538 scans). The following new resonances were observed: 104 ppm (intermediate), 107 ppm (a slowly formed side product which is an EPSP ketal[61]), 152 ppm (EPSP free in solution), 161 ppm (EPSP bound in a ternary complex with enzyme and phosphate), and 204 ppm (pyruvate formed from very slow enzymatic hydrolysis of EPSP). (Reprinted with permission from ref 54. Copyright 1990 American Chemical Society.)

to the enzyme. Before the isolation of the intermediate, ^{13}C NMR methods were employed to examine the species bound to the enzyme by using PEP enriched in ^{13}C at the C-2 position, on the basis of the expectation that there would be a shift in the ^{13}C resonance upon formation of the tetrahedral center. Previous chemical quench flow data had defined conditions under which the internal equilibrium could be formed with approximately 33% of the enzyme sites occupied by intermediate. Incubation of enzyme with S3P and [2-^{13}C]PEP resulted in the appearance of three new resonances, at 161, 107, and 104 ppm as shown in Figure 4.

The resonance at 104 ppm corresponded to the predictions for a carbon at a tetrahedral center bearing two oxygens. However, prior to the isolation of the intermediate, these results could *not* be interpreted unambiguously for two reasons. First, the chemical shift of a carbonium ion type intermediate would vary due to the degree of shielding at the active site, and therefore the chemical shift cannot be used to define the chemical identity of the intermediate. Second, even if one assumes that the resonance at 104 ppm is due to a tetrahedral center bearing two oxygens, this observation would not distinguish whether the second oxygen molecule was contributed by an enzyme nucleophile or by the shikimate ring. Thus, the observation of the NMR peak did not allow us to distinguish between the

two alternate mechanisms. For that reason, the results were uninterpretable and therefore unpublishable until after the intermediate was isolated and identified.

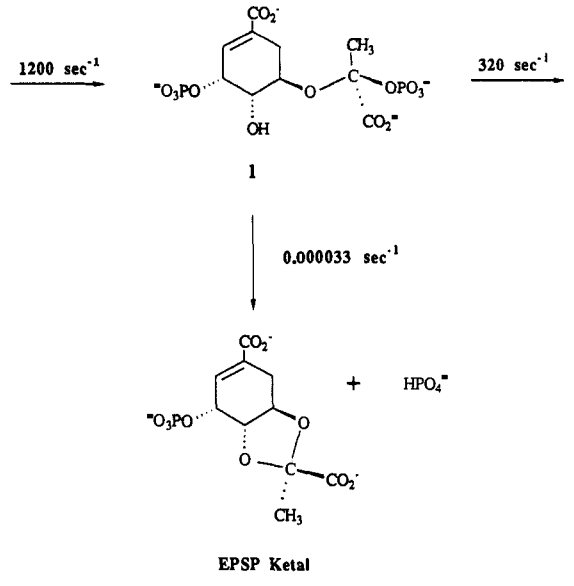
Once the intermediate was isolated and identified, the NMR spectrum of the material bound to the active site provided a confirmation of the rapid quench results. In fact, the two methods provide a perfect complement to one another. While examination by NMR allowed observation of all significant enzyme-bound species, some of which could have been missed in the quench experiments, the rapid quench methods provided for the identification and analysis of the time dependence of formation and decay of each species. This information was crucial to the rigorous interpretation of the NMR data in the case of EPSP synthase. Thus, only with the isolation and identification of the tetrahedral intermediate and the characterization of its NMR spectrum could the peak at 104 ppm in the spectrum of the material bound to the enzyme under internal equilibrium conditions finally be identified.[54]

In addition to the tetrahedral intermediate, two other novel peaks appeared in the ^{13}C NMR spectrum. According to the kinetic and equilibrium measurements, greater than 90% of the EPSP bound to enzyme would be in the form of a ternary complex with phosphate at the active site. The peak at 161 ppm was attributed to EPSP bound to the enzyme in a ternary complex with phosphate, on the basis of the following analysis, again dependent upon the need to account for species known to be bound to the enzyme at equilibrium. The chemical shift of free EPSP was determined to be 152 ppm in the absence of enzyme. Quantitation of the internal equilibrium by rapid quench methods demonstrated that nearly 60% of the sites were occupied by EPSP. In the initial NMR experiments, S3P and consequently EPSP were in excess over the enzyme, and so it was impossible to establish whether bound and free EPSP were both accounted for by the resonance at 152 ppm. If this were the case, then the peak at 161 ppm would represent a new chemical species. Therefore, on the basis of the knowledge of the enzyme kinetics and equilibria, an NMR experiment was designed with enzyme in excess over S3P such that essentially all of the EPSP formed would be bound to the enzyme in a ternary complex with phosphate. This was possible because the enzyme concentration of the NMR experiment (1.6 mM) greatly exceeded the dissociation constant for EPSP (1 μM) and because the overall equilibrium constant for the reaction was small (180) and the reaction could be driven backward by moderate concentrations of phosphate. Under these conditions, the peak known to be due to free EPSP was no longer observed, and therefore the remaining peak at 161 ppm had to be due to EPSP bound in a ternary complex with phosphate. The 9 ppm downfield chemical shift observed upon binding EPSP is quite large and may reflect the changes in the environment at the active site responsible for catalysis.

B. Identification of the EPSP Ketal, a Side Product

The third novel NMR peak, at 107 ppm, was observed only after several hours of scanning in the NMR (Figure 4d). Here one must clearly face the issue of whether the peak arises as a slow-forming product or

SCHEME VII. Formation of a Side Product



because of the time required to resolve the resonance from the noise, although, as shown in Figure 4c, it was clear that a high-quality spectrum which lacked this resonance could be obtained at early times. The confusion and the errors that can result when the rate of formation of a compound is overlooked are illustrated clearly in the paper published by Barlow et al.,[60] in which they claim to have seen a second form of the intermediate, a so-called enzyme-free intermediate. The compound giving rise to this unique resonance has been isolated by our co-workers at Monsanto and its structure established as the EPSP ketal shown in Scheme VII,[61] with only the *R* diastereomer being formed. An accurate measurement of the formation of the EPSP ketal established that it was a side product of the enzymatic reaction and *not* an enzyme-free intermediate. The rate of formation of the EPSP ketal, measurement by incubating enzyme and substrates under internal equilibrium conditions, was found to be 3.3×10^{-5} s^{-1}. Thus, the EPSP ketal is formed at a rate that is a millionfold slower than turnover of the tetrahedral intermediate as illustrated in Scheme VII. This analysis underscores the importance of kinetic information to distinguish enzymatic side products from true reaction intermediates.

It is important to ask whether the EPSP ketal is formed either in solution or at the active site of the enzyme because the answer may provide clues as to the chemistry at the active site and the geometry of the bound intermediate. The conditions for formation of the EPSP ketal parallel the conditions required to maintain the internal equilibrium. Thus, one might propose that the ketal is formed due to breakdown of the intermediate at a slow rate at the active site, or at a fast rate in solution following a slow release of the intermediate from the enzyme. The ketal is not formed from any of the substrates or products in the absence of enzyme. The ketal does form, however, from isolated intermediate in solution, but at a slow rate that is comparable to the rate of formation in the presence of enzyme, as described below.[56] At a superficial level, the observation that the rate of formation of ketal from the intermediate is the same in the presence or absence of enzyme might suggest that the ketal forms from the

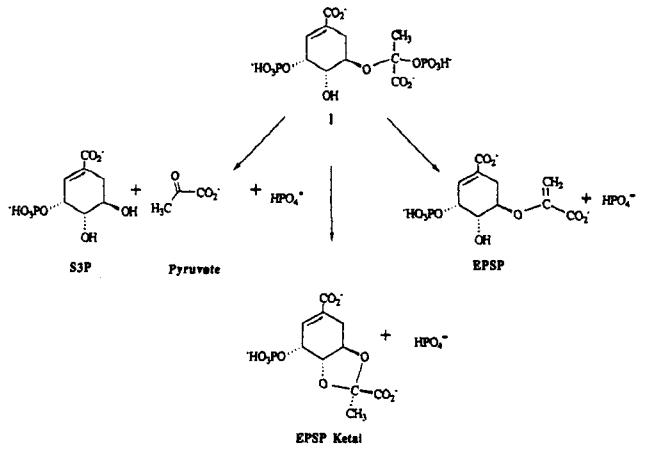


Figure 5. Breakdown of the tetrahedral intermediate in solution at pH 7.0. The product distribution was then determined by HPLC on a Mono-Q 5/5 anion-exchange column using a triethylammonium bicarbonate gradient (0.175–1.0 M) and monitoring with a continuous flow radioactivity detector. (Reprinted with permission from ref 56. Copyright 1990 Journal of Biological Chemistry.)

intermediate in solution. However, the rate of ketal formation in the presence of enzyme was measured from the internal equilibrium with enzyme in excess over substrates, and under these conditions the concentration of free intermediate is negligible (see below). Therefore, one is forced instead to the conclusion that the ketal must be formed from the intermediate at the active site of the enzyme, in a reaction which is not accelerated or inhibited by the binding to the enzyme. The rearrangement of the intermediate to form the ketal may represent the rare capture of an oxonium ion type intermediate (see Scheme XI) by the 4-OH, or it may be due to a direct in-line displacement of phosphate from the tetrahedral center by the 4-OH of S3P.

C. Kinetics of Rebinding of the Intermediate to the Enzyme

The isolation of a stable tetrahedral intermediate afforded us with a unique opportunity to examine its breakdown in solution and to establish the kinetics of its recombination with the enzyme. The rebinding of an intermediate to an enzyme is an important topic in enzymology and one that has not been possible to address by direct measurement. Nonetheless, the observed slow binding of transition-state and intermediate analogues has been used as a basis to argue that enzymes should normally bind the true intermediate or transition state very slowly,[62-66] although it is often the slow off-rate that governs the kinetics of "slow, tight-binding" inhibitors. With the intermediate in hand, this prevalent theory could be tested.

It was first necessary to determine the characteristics of the intermediate in solution. The stability of the intermediate in solution is markedly pH dependent with increasing rate of breakdown as the pH is lowered, in accord with expected pK_α values for protonation of the phosphate. However, at pH 7, the intermediate is quite stable, breaking down at a rate of 0.00026 s^{-1} (a half-life of 45 min). The yield of breakdown products from the intermediate in solution is shown in Figure 5. The intermediate decomposes in solution by elimination of phosphate with the products partitioning to form S3P (76%), EPSP (8%), and the EPSP ketal (16%) at pH 7. Given the percentage yield of ketal, the rate of its formation from the intermediate in solution is 4×10^{-5} s^{-1}.

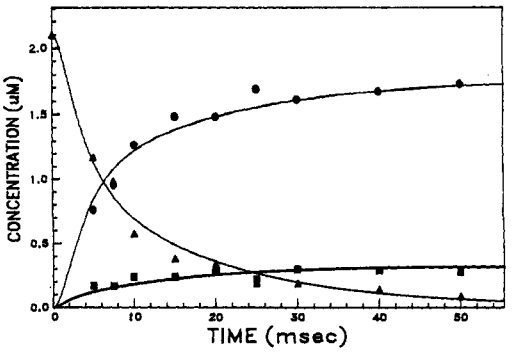
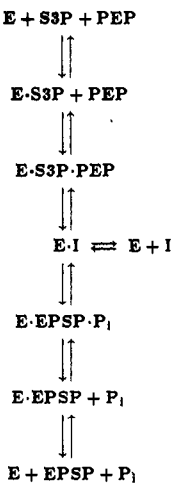


Figure 6. Intermediate rebinding to enzyme in a single turnover. Intermediate was synthesized with ^{14}C radiolabel in the carboxyl of the phospholactyl moiety as described above. Enzyme (10 μM) was mixed with intermediate (2.1 μM) for a specified time interval (5–50 ms), and the reaction was stopped by the addition of 0.2 M KOH (final concentrations) followed by vortexing with chloroform. The product distribution between intermediate (▲), EPSP (●), and PEP (■) was then determined by HPLC using the same chromatographic conditions described above. The curves were calculated by numerical integration with rate constants summarized in Scheme IV and using the program KINSIM,[49] as described previously. (Reprinted with permission from ref 48. Copyright 1988 American Chemical Society.)

SCHEME VIII. Binding of Intermediate



The first conclusion from these studies is that the enzyme must actively catalyze the decomposition of the intermediate and direct the formation of the desired products. It is not unreasonable to suggest that the elimination of phosphate is the rate-limiting step in both the enzyme-catalyzed and the solution decomposition of the intermediate. Accordingly, one can calculate that the enzyme accelerates the breakdown of the intermediate by 1.2 millionfold, suggesting that it contributes approximately 8 kcal/mol toward stabilization of the transition state for decomposition of the intermediate.

The rate of reaction of the intermediate with the enzyme was examined in a single-turnover experiment, where the intermediate was reacted with enzyme in excess to get the results shown in Figure 6. Surprisingly, the intermediate binds rapidly to the enzyme and partitions to form products in both forward and reverse directions for the enzyme-catalyzed reaction as illustrated in Scheme VIII. The data were fitted by simulation, adjusting only the rate at which the interme-

diate binds to the enzyme. The kinetics of its decomposition in partitioning to form products in both the forward and reverse directions were derived completely from the kinetics of the reactions established previously and summarized in Scheme IV. The second-order rate constant for binding of the intermediate to the enzyme was 5×10^7 M^{-1} s^{-1}. This rate is somewhat slower than the rates of binding S3P (6.5×10^8 M^{-1} s^{-1}) and EPSP (2×10^8 M^{-1} s^{-1}), but faster than the rate of binding of PEP (1.5×10^7 M^{-1} s^{-1}).

The enzyme site is able to accept the intermediate at rates comparable to the rates for binding substrates and products, even though it is presumably capable of binding quite tightly to the enzyme. Thus, the present direct measurement provides no support for the theory suggesting that intermediates should bind slowly to the enzyme.

D. Analogues of the Tetrahedral Intermediate

Analogues of reaction intermediates or transitions states have, in some instances, been observed to bind slowly to inhibit enzymes. Slowly is a relative term, and in this case, slowness is relative to rates of substrate binding and turnover. Inhibitors that fall into this class have been termed slow, tight-binding inhibitors to distinguish them from inhibitors in classical steady-state enzyme kinetics where the equilibrium between inhibitor and enzyme is assumed to be rapid.[63] The basis for the slowness of binding has been the topic of some interesting and reasonable conjecture. It has been postulated that analogues of transition states may bind slowly because the enzyme normally rests in a ground state. Thus, the transition-state analogue must either bind to a rare form of the enzyme or induce a change in conformation of the enzyme following initial, weak binding to the ground state. Either model can explain a slow rate of binding. Although the logic is correct, the question remains as to whether the slow binding is just a property of the analogue or whether this property is shared by the true transition state or intermediate. In a recent review comparing the kinetics for a large number of slow, tight-binding inhibitors, the emphasis again was on the rationale for the slow rate of binding,[67] but examination of the data comparing binding and dissociation rates of more than 30 inhibitors reveals that a large fraction of these compounds bind at rates exceeding 1×10^6 M^{-1} s^{-1}. Thus, perhaps a more universal characteristic of the slow, tight-binding inhibitors is their exceedingly slow off-rate which leads to a low K_d and slow net rate of binding at concentrations comparable to the K_d.

In the present case, it has been shown that the reaction intermediate binds at a fast rate, with a second-order rate constant of 50 μM^{-1} s^{-1}, and this provides a standard by which analogues of the intermediate might be compared. Bartlett and co-workers[68] have synthesized tight-binding analogues of the EPSP synthase tetrahedral intermediate by replacing the phosphate with a more stable phosphonate group. Both *R* and *S* stereoisomers were synthesized and shown to bind to the enzyme with inhibition constants of 0.015 and 1.13 μM, respectively. The binding and dissociation rates of the analogues were measured by stopped-flow fluorescence methods. Both analogues bind slowly to the enzyme, with second-order rate constants of 0.15 and 0.0027 μM^{-1} s^{-1} for the *R* and *S* isomers, respectively. Moreover, the binding of the *S* isomer was biphasic and suggestive of multiple steps leading to formation of the enzyme–inhibitor complex. The analogues dissociate at rates of 0.0022 and 0.0031 s^{-1}, respectively. It is reasonable to suggest that this rate may reflect a rate-limiting conformational change in the enzyme preceding release of the analogue and that the dissociation rate may be similar for the true intermediate. If that were the case, the K_d for the intermediate would be 0.05 nM, a value that is consistent with the measured free energy of the reaction at the active site of the enzyme relative to estimates of the free energy of formation of the intermediate in solution.[56] Thus, the analogues bind to the enzyme 2–4 orders of magnitude more *slowly* and more *weakly* than the true intermediate.

These results can be rationalized in terms of the structures of the analogues. The shortened carbon–phosphorus bond distances resulting from the removal of the oxygen atom apparently lead to substantial loss in interaction energy (3–6 kcal/mol). The binding of the analogues proceeds with substantial rearrangement of the protein to accommodate the unnatural structure, leading to a slow binding rate. According to this interpretation, the analogues fall short of the expected binding energy of the intermediate. The analogues still bind tightly relative to substrates because of the extremely tight binding of the intermediate that they are attempting to mimic. Their slowness of binding reflects the extent to which they fail in mimicking the intermediate, rather than representing a property of the intermediate itself. Thus, an analogue that bound to the enzyme with rates comparable to those of the true intermediate may be expected to bind even more tightly.

E. Stereochemistry of the Intermediate

The stereochemistry of the tetrahedral center of the intermediate is not currently known. Because the *R* isomer of the phosphonate analogue of the tetrahedral intermediate binds some 60-fold tighter to the enzyme than the *S* isomer, one might conclude that the stereochemistry at the tetrahedral center of the intermediate had been established as *R*. However, confidence in this assignment is greatly weakened due to the modest preference for the *R* isomer relative to the large difference between the affinities of the enzyme for the analogues as compared to the intermediate.[56] The free energies of binding for the intermediate, *R* analogues, and *S* analogue are −13.8, −10.5, and −8.0 kcal/mol, respectively (Table I). Thus, it is conceivable that the phosphonate analogues bind to the enzyme with reversal of the stereochemical preference since the shortened phosphorus to carbon bond lengths may allow the phosphonate to bind at the carboxylate site on the enzyme. Accordingly, one cannot derive with confidence the stereochemistry of the intermediate from the small difference in affinities for the two diastereomers of the phosphonate analogues.

The stereochemistry of the ketal might be used to argue for the stereochemistry of the intermediate, depending upon the mechanism of formation of the ketal. For example, if the ketal is formed by a backside attack of the 4-OH on the tetrahedral center with displacement

TABLE I. Binding Kinetics of Substrates, Intermediate, and Analogues[a]

	k_{on}, μM^{-1} s^{-1}	k_{off}, s^{-1}	K_d, μM	$-\Delta G°$, kcal/mol
S3P	650	4500	7.0	6.9
PEP	15	280	18.7	6.3
EPSP	200	200	1.0	8.0
P_i	0.07	100	1430	3.8
intermediate	50	(0.0025)	(0.000 05)	(13.8)
R isomer	(0.15)	0.0022	0.015	10.5
S isomer	0.0027	0.0031	1.13	8.0

[a] Rate and equilibrium constants for the binding of substrates, products, intermediate, and analogues are summarized as described previously.[48,51,56] The numbers without parentheses have been obtained by direct measurement, while those in parentheses are estimated. Measurements for the substrates and products are from Anderson et al.[48,51] The dissociation constants for the phosphonate *R* and *S* isomers are from Alberg and Bartlett.[68] Conditions: 50 mM HEPES, 50 mM KCl, pH 7.0, 20 °C.

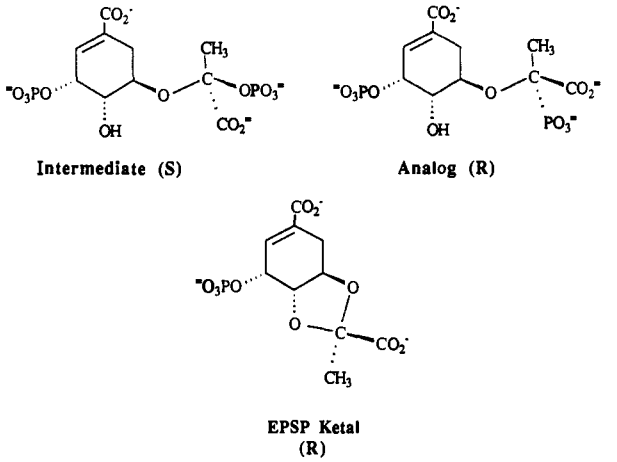


Figure 7. Stereochemical considerations for intermediate based upon EPSP ketal and phosphonate analogue.

of the phosphate, then there would be an inversion of the configuration. Accordingly, the observed *R* stereochemistry of the ketal would imply an *S* configuration of the tetrahedral intermediate (see Figure 7). However, once again, this analysis is subject to question. One could always argue that the ketal is formed by reaction of the 4-OH with an oxonium ion type species formed by loss of phosphate from the tetrahedral intermediate. According to this mechanism, the stereochemistry of the intermediate would be lost upon formation of the oxonium and the stereochemistry of the ketal could be due to a steric preference for the *R* isomer upon reaction with the 4-OH. Thus, two observations remain; each provides opposite and yet indirect and therefore equivocal information to suggest the stereochemistry of the intermediate. Perhaps only the crystallization of intermediate will resolve the issue.

F. Mechanism of Action of Glyphosate

The mechanistic questions surrounding the mode of action of glyphosate on EPSP synthase provide an important lesson with regard to targeted enzyme inhibition. Often, as was the case with glyphosate and EPSP synthase, the mechanism of an enzyme reaction is probed by screening for tight-binding inhibitors. Upon finding a tight-binding inhibitor, the assumption is then made that the inhibitor somehow resembles the enzyme transition state, and the synthetic effort is concentrated

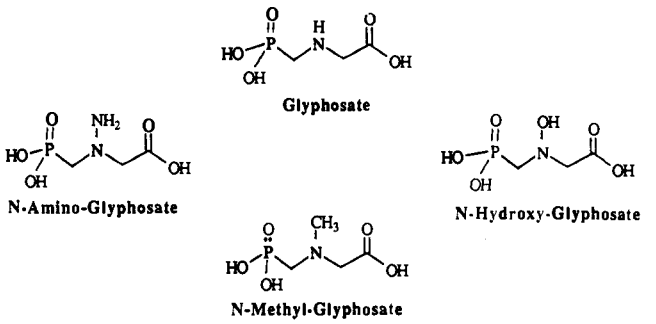


Figure 8. Glyphosate and derivatives that inhibit EPSP synthase.

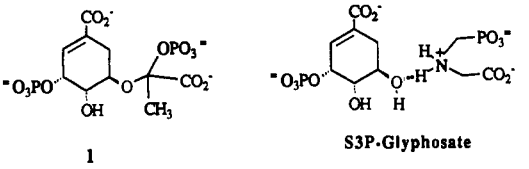


Figure 9. Comparison of intermediate to S3P–glyphosate complex.

on making derivatives and modified inhibitors. According to this logic, it has been suggested that glyphosate, in its protonated state, might be an analogue of the protonated transition state of PEP.[38,39] At Monsanto, numerous analogues of glyphosate were synthesized in the hopes of finding an even tighter binding inhibitor; however, only three glyphosate derivatives showed modest inhibition of EPSP synthase as shown in Figure 8.

The significant observation regarding the mode of action of glyphosate is that S3P and glyphosate combine at the active site to inhibit the enzyme. The most likely explanation is that the S3P–glyphosate complex resembles the overall geometry of the tetrahedral intermediate as shown in Figure 9 and glyphosate interacts with the same active-site residues which catalyze the formation and breakdown of the tetrahedral intermediate as illustrated in Scheme XI. In this complex, it is possible that the protonated amino group of glyphosate is hydrogen bonded to the 5-OH of S3P. Thus, one should consider the S3P–glyphosate complex an analogue of the tetrahedral intermediate, accounting for the specificity of glyphosate for EPSP synthase to the exclusion of other PEP-utilizing enzymes. A three-dimensional picture of S3P and glyphosate bound to the enzyme active site will provide a definitive answer to the question of the mode of enzyme interaction with this complex.

G. Crystal Structure of EPSP Synthase

Preliminary results on the crystallization of the *Escherichia coli* EPSP synthase were reported in 1985;[69] however, the conditions employed yielded crystals having rather large unit cell dimensions, a = 124, b = 124, and c = 381, which were unsuitable for high-resolution X-ray analysis. An in-depth analysis of these crystals and crystallization conditions showed that an inactive, oxidized form of the enzyme had been crystallized.[70] By using a modification of the crystallization conditions and highly purified EPSP synthase, Nancy Leimgruber (Monsanto) was able to obtain high-quality crystals suitable for high-resolution X-ray analysis. These crystals have lattice constants of a =

TABLE II. Definitions of Steady-Steady Kinetic Parameters[a]

Forward Reaction

$$k_{cat} = k_p k_5 k_6/(k_5 k_6 + k_{-p} k_6 + k_p k_6 + k_p k_5)$$
$$K_{m,S3P} = k_p k_5 k_6/(k_1(k_5 k_6 + k_{-p} k_6 + k_p k_6 + k_p k_5))$$
$$K_{m,PEP} = k_6(k_p k_5 + k_{-2} k_5 + k_{-2} k_{-p})/(k_2(k_5 k_6 + k_{-p} k_6 + k_p k_6 + k_p k_5))$$

Reverse Reaction

$$k_{cat} = k_{-1} k_{-2} k_{-p}/(k_{-2} k_{-p} + k_{-1} k_{-p} + k_{-1} k_p + k_{-1} k_{-2})$$
$$K_{m,EPSP} = k_{-1} k_{-2} k_{-p}/(k_{-6}(k_{-2} k_{-p} + k_{-1} k_{-p} + k_{-1} k_p + k_{-1} k_{-2}))$$
$$K_{m,P_i} = k_{-1}(k_p k_5 + k_{-2} k_5 + k_{-2} k_{-p}/(k_{-5}(k_{-2} k_{-p} + k_{-1} k_{-p} + k_{-1} k_p + k_{-1} k_{-2}))$$
$$k_p = k_3 k_4/(k_{-3} + k_4) \qquad k_{-p} = k_{-3} k_{-4}/(k_{-3} + k_4)$$

[a] The definitions of the steady-steady kinetic constants for EPSP synthase are given in terms of the intrinsic rate constants for the pathway. Net rates of conversion of E·S3P·PEP ⇌ E·EPSP·P_i are defined by k_p and k_{-p}, in the forward and reverse directions, respectively.

$$E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S3P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}S3P{\cdot}PEP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E{\cdot}I \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E{\cdot}EPSP{\cdot}P_i \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}}$$
$$E{\cdot}EPSP \underset{k_{-6}}{\overset{k_{-6}}{\rightleftharpoons}} E$$

TABLE III. Values for Steady-State Kinetic Parameters[a]

parameter	measured	calculated
	Forward Reaction	
k_{cat}, s^{-1}	60	60
$K_{m,S3P}$, μM	(7)	0.09
$K_{d,S3P}$, μM	7	7
$K_{m,PEP}$, μM	8	6
	Reverse Reaction	
k_{cat}, s^{-1}	10	12
$K_{m,EPSP}$, μM	(2)	0.06
$K_{d,EPSP}$, μM	2	1
K_{m,P_i}, mM	2	1.5

[a] The measured values for the steady-state kinetic parameters[46,47] are compared to the values calculated from the equations given in Table II according to the intrinsic rate constants summarized in Scheme IV. Because of the rapid rates of binding and dissociation of S3P and EPSP, the steady-state kinetics follow a rapid equilibrium ordered bi-bi pathway. Accordingly, the K_m's for S3P and EPSP are too small to measure and drop out of the steady-state velocity equation,[73] and the measured, apparent K_m values, shown in parentheses, correspond to the K_{ia} terms which equal the dissociation constants for S3P and EPSP, $K_{d,S3P}$ and $K_{d,EPSP}$, respectively (see text).

92, b = 82, and c = 72, with one molecule in the asymmetric unit.[71] The purity and catalytic activity of these crystals were established by HPLC analysis and soaking experiments using radiolabeled substrates.[70] The rapid solution of the X-ray crystal structure of EPSP synthase after the high-quality crystals were obtained demonstrates the importance of concerted efforts in biochemistry and crystallography and that the rate-limiting step in obtaining a protein crystal structure is usually the availability of high-quality crystals.[72]

At 3-Å resolution, the protein shows a rather unusual folding pattern with two approximately equal globular domains connected by two crossover segments of the polypeptide backbone.[71] The protein resembles an open clam, leading to the obvious suggestion that the enzyme may close up upon the binding of substrates. If this were true, it would imply an induced fit type mechanism with substantial rearrangement of the protein following the binding of substrates or the intermediate. A structure at higher resolution will be required to substantiate this suggestion. We eagerly await the structure showing S3P and glyphosate bound to the enzyme active site.

IV. Kinetic and Thermodynamic Analysis of the Reaction Pathway

The complete analysis of the reaction catalyzed by EPSP synthase led to the estimates for all 12 rate constants along the reaction sequence plus those governing the binding and dissociation of the intermediate. When such complete understanding of the reaction pathway is reached, one of the first questions to address is whether the individual kinetic constants can quantitatively account for the steady-state kinetic parameters. The measured and calculated steady-state parameters for both the forward and reverse reactions are summarized in Tables II and III. It is important to recognize that the rapid binding and dissociation of S3P and EPSP lead to a "rapid equilibrium ordered bi bi" steady-state mechanism. As noted in Table III, the calculated K_m values (0.09, 0.06 μM) are substantially lower than the measured K_m (7, 2 μM) and K_d (7, 1 μM) values for S3P and EPSP, respectively. However, this apparent discrepancy can be reconciled by an understanding of the nature of a rapid equilibrium, ordered bi-bi steady-state mechanism. The steady-state velocity expression for a complete ordered bi-bi mechanism is

$$\frac{v}{V_{max}} = \frac{[A][B]}{K_{ia}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]}$$

but for a *rapid equilibrium*, ordered bi-bi mechanism, the magnitude of K_{mA} is negligible, leading to the simplified expression[73]

$$\frac{v}{V_{max}} = \frac{[A][B]}{K_{ia}K_{mB} + K_{mB}[A] + [A][B]}$$

For a rapid equilibrium mechanism, the apparent K_m for substrate A is defined by the parameter K_{ia} which equals the true K_d.[73] Thus, for EPSP synthase, the terms containing the K_m values for S3P and EPSP in the forward and reverse directions, respectively, drop out of the steady-state velocity expression, and the apparent, measured K_m values for S3P and EPSP actually correspond to the dissociation constant for each substrate. Given this understanding of the reaction kinetics, there is good agreement between the measured and calculated steady-state kinetic constants.

A. Free Energy Profile

Free energy diagrams for the reactions occurring at the active site of EPSP synthase are shown in Figure 10, including a comparison to the same reactions occurring in solution. This free energy profile was calculated for the approximate physiological concentrations of substrates and products as described in the legend to the figure. Under these conditions, the steady-state concentration of the intermediate is estimated to be 0.24 nM by simultaneous solution of the equations describing the enzyme-catalyzed reaction including the release and binding of the intermediate. Thus, one perhaps surprising conclusion of these studies is that the intermediate, although tightly bound to the enzyme, must reach a finite steady-state concentration in vivo. The steady-state concentration is larger than the K_d for the intermediate, largely due to the compe-

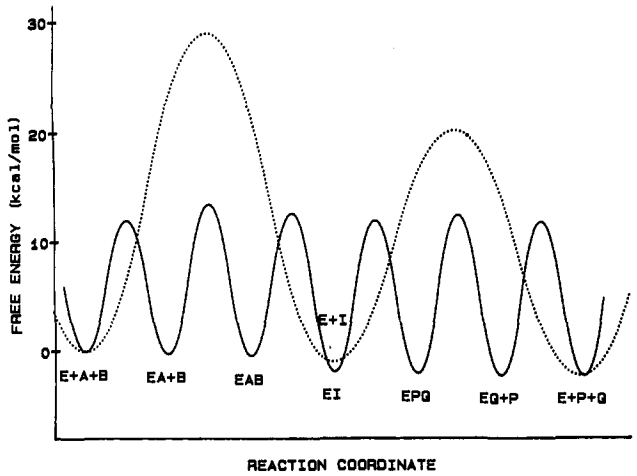


Figure 10. Free energy diagram for EPSP synthase. The free energy diagram was calculated from the rate constants measured previously[48,56] by comparing the reaction in solution (dotted line) to the enzyme-catalyzed reaction (solid line). Apparent free energies of activation were calculated from transition-state theory: $\Delta G^{\circ\ddagger} = -RT \ln (k_{obsd} + RT \ln (kT/h))$. The free energy for formation of the intermediate in solution was calculated as described previously.[56] The rate of breakdown of the intermediate in solution was used to calculate the free energy of activation for the conversion of intermediate to EPSP and P_i. The transition state in forming the intermediate from S3P and PEP in solution was calculated on the basis of a reasonable upper estimate of a 10^{12}-fold acceleration of the rate by the enzyme. Free energies were calculated for the approximate physiological concentrations 10 μM S3P (A), 25 μM PEP (B), 1 mM P_i (P), and 1 μM EPSP (Q). Simultaneous solution of the equations describing enzyme turnover and the binding of the intermediate allowed calculation of the concentration of intermediate present at steady state. At the concentrations of substrates and products given above, we estimate a concentration of 0.24 nM intermediate, which was then used in construction of the free energy diagram. Conditions: 20 °C, pH 7.0. (Reprinted with permission from ref 48. Copyright 1988 American Chemical Society.)

tition for enzyme sites by the binding of the substrates and products of the reaction.

The most striking feature of this diagram (Figure 10) is the extent to which the two large activation barriers for the reactions in solution have been replaced by a series of smaller barriers in the enzymatic pathway, leading to efficient catalysis. The free energy change for the overall reaction is small, −2.2 kcal/mol (ΔG° = −3.0 kcal/mol). The active-site reactions, E·S3P·PEP → E·I → E·EPSP·P_i, occur with free energy changes of −1.4 and −0.2 kcal/mol, respectively. Thus, a large fraction of the total free energy change for the reaction occurs upon formation of the intermediate at the active site.

One must be careful to note that the free energies for the formation and breakdown of the intermediate in solution depend upon the choice of standard state, whereas the corresponding values for reactions at the active site do not. Thus, one must take care to compare the values in solution with those at the active site only under a defined set of conditions and to consider the entropic effects on binding. While Figure 10 shows the complete free energy profile under physiological conditions, in Figure 11 we illustrate the free energy changes under standard-state conditions (1 M, pH 7.0), focusing on the reactions involving the conversion of *substrates → intermediate → product* at the enzyme active site and in solution. This comparison highlights the role of the enzyme in catalyzing the formation and

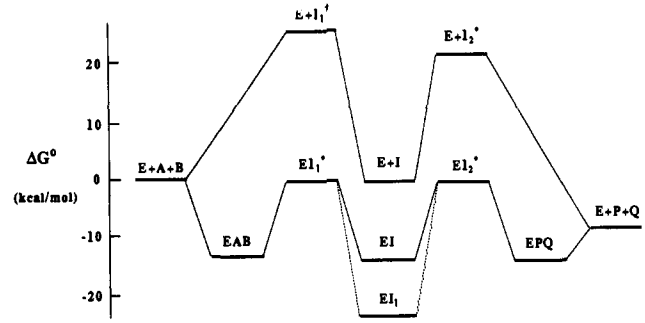
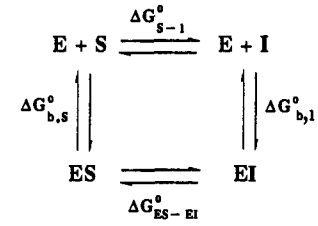
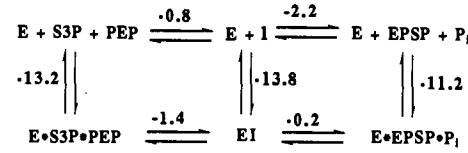


Figure 11. Standard-state free energy diagram for EPSP synthase. An abbreviated standard-state free energies profile is shown without apparent activation barriers for the steps involving the binding and release of substrates, products, and the intermediate. The free energies are calculated by using the kinetic constants as in Figure 10, but under standard-state concentrations (1 M, at 20 °C, pH 7.0). The theoretical intrinsic binding energy used to define the state, EI_i, was estimated from the sum of the binding energies of the two substrates plus a term for the entropy effect expected in binding one intermediate as compared to two substrates, −9 kcal/mol. The actual intrinsic binding energy for the intermediate may be greater to the extent that the binding of the substrates is subject to strain that weakens their interaction. The diagram illustrates, to a first approximation, the extent to which the enzyme accelerates the reaction while destabilizing the intermediate.

SCHEME IX



SCHEME X



decomposition of the intermediate.

The observed binding energy for the intermediate is related to its intrinsic binding energy and its free energy of formation from S3P and PEP at the active site by a thermodynamic box as illustrated in Scheme IX. Here we simplify the diagram by using one *S*, which is intended to represent the sum of the binding of the two reactants. The contributions toward the observed free energy of binding the intermediate, $\Delta G_b^\circ{}_I$, can be interpreted in terms of the free energy of binding the substrate, $\Delta G_b^\circ{}_S$, and the difference in free energy for the chemical reaction in solution and at the active site according to the thermodynamic box describing the reactions as shown in Scheme IX. We define a term, $\Delta\Delta G^\circ$, to represent the differences in binding energy between the substrate and intermediate which is equal to the difference in free energy of the reaction in solution and at the active site.

$$\Delta\Delta G^\circ = \Delta G_b^\circ{}_I - \Delta G_b^\circ{}_S = \Delta G^\circ{}_{ES=EI} - \Delta G^\circ{}_{S=I} = -0.6 \text{ kcal/mol}$$

This difference, which reflects the extent to which the

enzyme has changed the free energy for formation of the intermediate, is rather small, −0.6 kcal/mol, under standard-state conditions (1 M, pH 7.0) as summarized in Scheme X. The numbers on the scheme have units of kilocalories per mole.

It is useful to estimate the magnitudes of the terms that contribute to the net difference in standard-state free energy. The difference in free energy of binding between substrates and intermediate can be broken down theoretically into the intrinsic binding energy difference, $\Delta\Delta G_b°_{intrinsic}$, and the difference in the amount of energy that is used to selectively stabilize or destabilize the substrates or intermediate, $\Delta\Delta G°_{strain}$. Thus, one can write

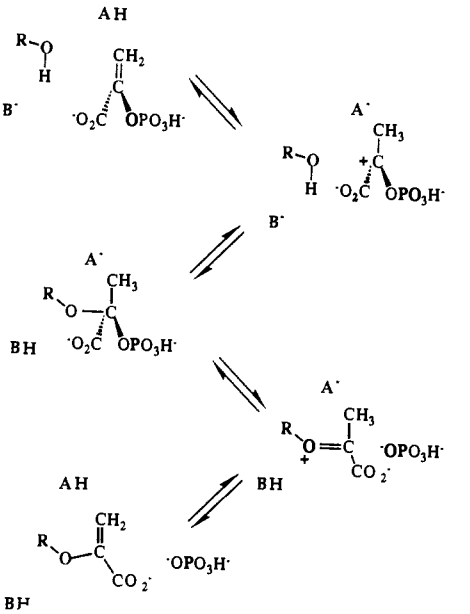
$$\Delta\Delta G° = \Delta\Delta G°_{intrinsic} - \Delta\Delta G°_{strain}$$

To a first approximation, one might expect that the enthalpic terms contributing to the intrinsic binding of substrates and intermediate should be similar. On the other hand, there is likely to be a large contribution to $\Delta\Delta G°_{intrinsic}$ from differences in the entropy loss that occurs upon binding two substrates.[10] For a bimolecular reaction such as that catalyzed by EPSP synthase, the difference due to rotational and translational entropy in comparing the binding of one intermediate versus two substrates can be quite large and may contribute as much as ~9 kcal/mol toward stabilization of the intermediate.[10,35] Thus, to a first approximation, $\Delta\Delta G°_{intrinsic} \simeq -9$ kcal/mol. If this were the case, the intermediate would bind very tightly to the enzyme with a free energy approaching −23 kcal/mol as shown in Figure 11, where EI_i represents the theoretical enzyme–intermediate state obtained from the expected intrinsic binding energy. However, it is evident that the enzyme *destabilizes* the intermediate by the amount necessary to cancel this large entropic effect. That is, to prevent the intermediate from falling into a deep thermodynamic pit, the enzyme has evolved to use the intrinsic binding energy toward the destabilization of the intermediate, rather than realize all available energy by tighter binding.

The extent of destabilization of the intermediate by the enzyme is also manifested in the degree to which the enzyme accelerates its decomposition. The enzyme catalyzes the decomposition of the intermediate by a factor of 1.2×10^6, corresponding to approximately 8.2 kcal/mol of transition-state stabilization energy. This analysis again suggests that the intrinsic binding energy of the enzyme for the transition state leading to decomposition of the intermediate should be quite large, −22 kcal/mol, predicting a dissociation constant in the subfemtomolar range. It may be unlikely that any transition-state analogue could approach such binding affinity; even one that failed by many orders of magnitude would be a potent inhibitor.

A single catalytic cycle of EPSP synthase is rather remarkable. The enzyme must first recognize the substrates and bind them sufficiently tightly to constrain them at the active site. Then, by selective stabilization of the first transition state, it catalyzes the formation of the intermediate. Finally, by selective destabilization of the intermediate and stabilization of the second transition state (for decomposition of the intermediate), the enzyme catalyzes the formation of the products, which are then released from the active site.

SCHEME XI



B. Active-Site Mechanism: Concerted or Stepwise?

It is instructive to consider for a moment the reaction sequence in Scheme XI, which shows a hypothetical stepwise reaction mechanism. This scheme postulates that an acidic residue at the active site first serves to protonate the PEP, leading to a highly reactive carbonium ion. This species then reacts in the second step with the 5-OH of S3P to form the tetrahedral intermediate; an active-site base removes the proton from the 5-OH, activating it for this nucleophilic reaction. The breakdown of the intermediate to form EPSP with the elimination of phosphate could follow a similar two-step reaction sequence via a reactive oxonium species as shown.

An alternative to this scheme would propose these reactions occur in a *concerted* fashion, rather than the *stepwise* mechanism shown, although the same active-site residues may participate. According to the stepwise reaction, the formation of the carbonium ion would be rate limiting and would occur at the observed rate of 1200 s^{-1}; the carbonium ion would react with the 5-OH at a rate of at least 10^{10} s^{-1}.[10] Thus, the carbonium ion intermediate would never accumulate and the two pathways, concerted versus stepwise, would be indistinguishable kinetically.

The question of whether the reaction occurs in a concerted or a stepwise sequence can be addressed by thermodynamic constraints. Although it may be reasonable to propose a carbonium ion formed by the protonation of PEP, it is considerably less favorable to form the same carbonium ion species by the reverse reaction, which would proceed by elimination of S3P–OH from the tetrahedral intermediate. Thus, the need for microscopic reversibility would tend to argue in favor of a concerted reaction mechanism. Nonetheless, the transition state may share some properties of the carbonium or oxonium species dependent upon the extent of charge transfer as the reaction progresses. Thus, if the charged species exist as unstable intermediates, they resemble the transition state, in accord with the Hammond postulate.[74]

The value of considering this pathway is that it provides reasonable suggestions for the roles of active-site residues in catalysis and it allows an explanation of the proton-exchange reactions observed in the presence of dideoxy-S3P (see Scheme III). Accordingly, the exchange of the methylene protons of PEP can occur in the absence of the 5-OH of S3P by the protonation of the PEP to form a carbonium ion intermediate. In the absence of the 5-OH, the active-site base may move into a position to help stabilize the carbonium ion. Thus, although the carbonium ion species would not accumulate in the presence of dideoxy-S3P, it could be stabilized sufficiently to account for the relatively slow exchange process. The rate of the exchange reaction in the presence of dideoxy-S3P is approximately 10 000-fold slower than the reaction to form the intermediate at the active site, and so only a modest stabilization of the carbonium ion may account for the results of Anton et al.,[41] with the simple addition–elimination mechanism without the need to postulate an active site nucleophile based mechanism.

C. Definitions of Kinetic Competence

According to the classical steady-state methods, "kinetic competence" of an enzyme intermediate has been addressed by asking whether a postulated intermediate reacts with the enzyme at a rate sufficient to account for the normal rate of conversion of substrate to product in the steady state. However, this definition of kinetic competence leads to significant confusion and difficulty in interpretation because it is defined in terms of a reaction that may be limited by the kinetics of binding of the intermediate to the enzyme. In the normal course of the enzyme reaction, the intermediate rarely leaves the enzyme active site and a step involving binding of the intermediate is not on the normal reaction pathway. In those cases where the binding is slow, the steady-state tests for kinetic competence do not provide unambiguous data to support the identification of the intermediate in the reaction. Studies on EPSP synthase call into question the standard steady-state definitions of kinetic competence of enzyme intermediates and point to the importance of the transient-state definition for kinetic competence. The kinetic competence of the EPSP synthase tetrahedral intermediate has been established unequivocally by single turnover experiments to measure the rates of its formation and decomposition in both the forward and reverse directions.

Analysis of the reactions catalyzed by aminoacyl-tRNA synthetases also illustrates the importance of establishing the kinetic competence of a potential intermediate by direct measurement. Aminoacyl-tRNA synthetase catalyzes two sequential reactions

$$\text{ATP} + \text{aa} \rightleftharpoons \text{aa-AMP} + \text{PP}_\text{i}$$

$$\text{aa-AMP} + \text{tRNA} \rightleftharpoons \text{aa-tRNA} + \text{AMP}$$

where aa represents an amino acid that reacts with ATP to form the aminoacyl adenylate (aa-AMP). The first reaction forms the aa-AMP and holds it tightly to the enzyme, and then tRNA reacts to form aa-tRNA. In an elegant and direct series of experiments, the kinetic competence of aa-AMP as a true reaction intermediate has been established.[35,36] In this case, the existence of aa-AMP was not questioned because it could be easily formed in the absence of tRNA. Rather, the issues centered on whether the aa-AMP was formed in the presence of tRNA. The reaction of aa-AMP with tRNA is rather slow (4 s^{-1}), and the kinetic competence of aa-AMP was established by direct measurement.[3]

In other cases, tests for kinetic competence of isolated or synthetic intermediates have been performed in the steady state.[75] For example, formyl phosphate has been proposed as an intermediate in the reaction catalyzed by N^{10}-formyltetrahydrofolate synthetase.[21,22] Formyl phosphate has been synthesized and reacts with THF to form N^{10}-formyl-THF, but *only* in the presence of ADP and at a maximum rate of 5 s^{-1}, which is 30-fold slower than k_{cat} for the normal substrates, although a burst of product formation occurs when the enzyme is first preincubated with ADP and formyl phosphate.[22] These data suggest that the binding of formyl phosphate is rate limiting, representing a step which is not on the normal pathway of reaction. Moreover, all three substrates must be present for optimal rates of reaction to occur, and so the binding of THF has an apparent synergistic effect in enhancing the rate of reaction of ATP with formate.[22]

Cleland recently has presented a simplified analysis relating the kinetics of binding and release of enzyme intermediates to their free energies of formation from substrates on the enzyme and in solution. The basis for this analysis is simply that the equilibrium constant for binding of an intermediate, and therefore the ratio of its on- and off-rates, can be derived from thermodynamic box arguments from a knowledge of the equilibrium constants for the binding of substrates and for the conversion of the substrates to intermediate at the active site and in solution as we described above and outlined in Scheme X. Thus, if an enzyme greatly shifts the equilibrium toward formation of the intermediate, then it must bind the intermediate much more tightly than it binds the substrates; this, in turn, puts limits on the magnitude of the rate constants governing the binding and release of the intermediate.

This analysis does not provide answers to mechanistic questions regarding the basis for the slow binding of some putative intermediates. Certainly, the thermodynamic analysis is correct in relating the K_d of the intermediate to its free energy of formation in solution and at the active site of the enzyme. However, there are several limitations of the analysis largely stemming from the difficulty and uncertainty in estimating the free energy for formation of the intermediate in solution. Moreover, the analysis is misleading in that it assumes that the calculated K_d of the intermediate is equal to the ratio of the apparent off-rates and apparent binding rates. For examples, it is quite likely that upon reaction of formyltetrahydrofolate synthetase with ADP, formyl phosphate, and THF, the normal order of addition of substrates to the enzyme may lead to a reaction sequence with the binding of ADP, then formyl phosphate, and then THF. Accordingly, the maximum rate of turnover of formyl phosphate may be limited by the rate of its binding to E·ADP, by the rate of binding THF to E·ADP·formyl-phos, or by the rate of a conformational rearrangement of the quaternary E·ADP·formyl-phos·THF complex. The least likely explanation is that the reaction occurs by the simple ad-

dition of formyl phosphate to the E·ADP·THF complex. Thus, calculation of the off-rate for the intermediate from the thermodynamic estimations and the *apparent* on-rate cannot be justified. In many cases, measurement of the rates of reaction at the enzyme active site may be more simple and direct.

In studies on ribulose 1,5-bisphosphate carboxylase, Calvin originally proposed a 2-carboxy-3-keto intermediate formed by reaction of CO_2 with ribulose 1,5-bisphosphate.[18] This intermediate has recently been isolated following rapid quench of an enzyme–substrate mixture by Pierce et al.[19,20] When the keto form of the intermediate is added back to the enzyme, it reacts at a rate of 0.07 s^{-1}, compared to k_{cat} of 3 s^{-1} observed with the normal substrates. Thus, this intermediate fails the steady-state test for kinetic competence. In the normal reaction, the keto form of the intermediate must be hydrated and then cleaved to yield two molecules of 3-phosphoglycerate. Therefore, two explanations exist for the slow reaction of the ketocarboxylated species: either the binding of the intermediate from solution is slow and rate limiting or the hydration of the keto form of the intermediate limits its rate of reaction. According to the second explanation, the hydrated intermediate may be formed directly in a concerted reaction involving the addition of both CO_2 and H_2O to ribulose 1,5-bisphosphate, and so the keto form may not be a kinetically significant intermediate on the reaction pathway.

For both enzymes, the data demonstrate the limitations of the steady-state tests for kinetic competence, which ask whether a postulated intermediate can bind to the enzyme fast enough to account for rates of turnover. Only one conclusion is certain: the binding of the intermediate to the enzyme is not on a step on the normal reaction pathway.

V. Summary

Transient-state kinetic analysis can provide direct evidence describing the reactions occurring at enzyme active sites. In the case of EPSP synthase, single-turnover experiments established the kinetic competence of the intermediate and defined the conditions under which it could be formed at the enzyme active site and then isolated. The moderate stability of the tetrahedral intermediate in solution at neutral pH and the availability of structurally similar analogues provided a splendid opportunity to test theories on the kinetics of binding of intermediates and their analogues to enzyme active sites. These studies point to the need for rigorous definitions of kinetic competence in terms of reactions occurring at enzyme active sites and call into question the significance of the slow binding observed for many analogues of enzyme intermediates. Moreover, they point to the exquisite complementarity of the enzyme for binding substrates and intermediates to achieve a proper balance between the optimal rates of binding substrates and releasing products and yet catalyzing their interconversion by selective stabilization of the transition states.

VI. Abbreviations Used

Dideoxy-S3P, 4,5-dideoxyshikimic acid 3-phosphate; EPSP, 5-enolpyruvoylshikimate 3-phosphate; glyphosate, *N*-(phosphonomethyl)glycine; S3P, shikimate 3-phosphate; PEP, phosphoenol pyruvate; P_i, inorganic phosphate; THF, tetrahydrofolate.

VII. Acknowledgments

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VIII. References

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