Detection and Identification of Transient Enzyme Intermediates Using Rapid Mixing, Pulsed-Flow Electrospray Mass Spectrometry†

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ABSTRACT: Rapid chemical quench methods coupled with off-line detection have proven to be very useful in identifying enzyme reaction intermediates. However, a limitation to this approach involves enzyme intermediates which are too labile under the chemical quenching conditions to allow detection and characterization. In this report, we describe the development of a novel approach for the detection and characterization of enzyme intermediates on the subsecond time scale using a "pulsed flow" method which employs a direct interface between a rapid-mixing device and electrospray ionization mass spectrometry. The application of this technique with the enzyme 5-enolpyruvoyl-shikimate-3-phosphate (EPSP) synthase is demonstrated. This enzyme converts shikimate-3-phosphate (S3P) and phosphoenol pyruvate (PEP) to EPSP and inorganic phosphate. Previous rapid chemical quench studies have shown that this reaction proceeds through a tetrahedral intermediate [Anderson, K. S., *et al.* (1988) *J. Am.Chem. Soc*. *110*, 6577−6579] formed transiently at the enzyme active site. We have shown that this tetrahedral intermediate can be directly detected on a subsecond time scale *without chemical quenching* by interfacing a rapid mixing apparatus directly with an on-line electrospray ionization ion trap mass spectrometer. Negative ion mass spectra collected by electrospray ionization indicate peaks for S3P (*m/z* 253), PEP (*m/z* 167), EPSP (*m/z* 323), and the tetrahedral intermediate (*m/z* 421). Further confirmation was provided by performing the same experiment with [^{13}C-1]-labeled PEP. These spectra confirmed the anticipated shift of 1 atomic mass unit for PEP (*m/z* 168), EPSP (*m/z* 324), and the tetrahedral intermediate (*m/z* 422) with no change in S3P (*m/z* 253). The collision-induced dissociation of the unlabeled tetrahedral intermediate peak (*m/z* 421) produced a daughter ion at *m/z* 323, which is most likely EPSP resulting from the loss of phosphate and is consistent with previous studies which have examined the chemical breakdown of the tetrahedral intermediate in solution [Anderson, K. S., *et al.* (1990) *J. Biol. Chem. 265*, 5567−6672]. This technique is under development and should be a useful method to study the transient formation of enzyme intermediates.

Protein structure function studies and biorational inhibitor design depend upon a detailed understanding of how chemical catalysis occurs at the enzyme active site. Key insights into this process are provided by detection of transient enzyme reaction intermediates which establish the catalytic reaction pathway. A transient kinetic approach using rapid chemical quench techniques has proven to be very useful for the direct observation of enzyme reaction intermediates (*1*−*4*). One of the limitations with this technique, however, is that a putative reaction intermediate may be chemically labile and hence not isolable under the experimental conditions required to terminate the enzymatic reaction. An alternative approach for the observation of transiently stable enzyme intermediates involves the use of electrospray ionization mass spectrometry (ESIMS).[1] Previous studies using ESIMS have shown that chemical reaction kinetics and enzymatic reactions can be monitored using either continuous flow methods or chemical quenching followed by direct injection (*5*−*10*). These methods, however, preclude the detection of short-lived chemical species which have half-lives in the 100 ms time range and hence would be converted to products during the time required for continuous flow injection or the study of intermediates that are labile under quenching conditions.

In this report, we describe the development of a novel method that directly interfaces a rapid-mixing apparatus in a "pulsed-flow mode" with ESIMS. A modified rapid-mixing apparatus and ESI interface are used to generate a pulsed flow in milliseconds which is amenable to ESI ion trap mass spectrometry. This method circumvents a requirement for chemical quenching and provides the additional advantage of rapid, on-line detection using the high-resolution, accurate and sensitive technique of mass spectrometry. The general premise of the rapid-mixing, pulsed-flow ESI method involves the use of a computer-controlled stepping motor which allows precise control of the speed at which solutions of enzyme and substrate are mixed. The enzyme and substrate solutions come together in a mixing tee which has a short fused silica capillary column on the outlet. The

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[1] Abbreviations: PEP, phosphoenol pyruvate; S3P, shikimate-3-phosphate; EPSP, 5-enolpyruvoyl-shikimate-3-phosphate; ESIMS, electrospray ionization mass spectrometry; CID, collision-induced dissociation; amu, atomic mass unit.

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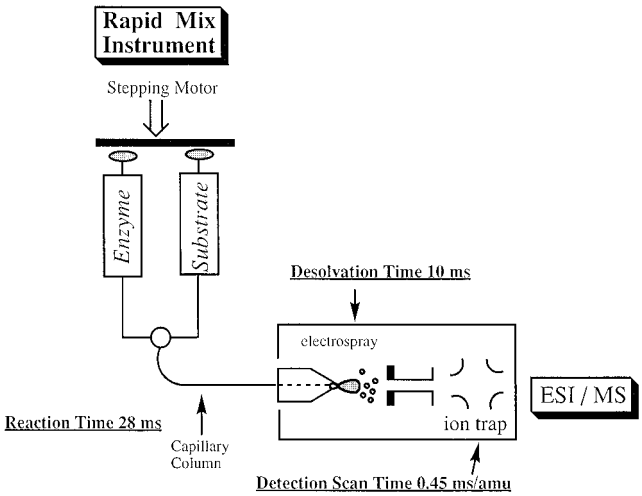


FIGURE 1: Schematic illustration of the rapid mix, pulsed-flow ESI instrument.

capillary leads directly to the electrospray unit in the mass spectrometer. The high voltage on the electrospray unit disperses the solution coming through the capillary into small particles for nebulization. At this point, rapid desolvation occurs in approximately 10 milliseconds and the enzyme reaction is terminated. The ions pass into the ion trap where they are detected in a scanning mode ~0.45 ms/amu (45 ms/100 amu). The extent to which the enzyme reaction proceeds is a function of both the length of the capillary column and the speed at which the solutions are mixed. The reaction time can be varied by driving the solutions together at different rates through the capillary before terminating in the electrospray process. In this work, we demonstrate that ESIMS can be used to directly detect transiently stable enzyme intermediates using a pulsed-flow interface on a subsecond time scale.

EXPERIMENTAL SECTION

Apparatus. A schematic illustration of the instrumentation used for this work is shown in Figure 1. A Kintek RFQ-3 rapid chemical quench apparatus (Kintek Instruments, State College, PA), which allows one to examine reaction times from 3 ms to multiple seconds, was used for these experiments (*11*). This apparatus was modified to accept two 0.5 mL syringes. The standard 8-way valve and sample loading valves were removed from the apparatus. In order to minimize any sample leaking, the Teflon syringe adapters were replaced with gas-tight Kel-F adapters which were connected to a zero dead volume mixing tee (Upchurch Scientific Co.) using PEEK tubing. The Kel-F adapters were hand-machined to fit the 0.5 mL syringes. An 11 cm fused capillary column (0.01 cm i.d.) provided the connection from the outlet of the mixing tee to the electrospray unit of the mass spectrometer. Solutions of enzyme and substrates were loaded into the syringes of the apparatus before connection to the mixing tee. The computer-controlled interface which drives the stepping motor was operated in manual mode to select the speed and volume of the each pulse of sample. For the experiments described in this report, the speed of the motor was between 32 and 128 rpm. Accordingly, driving the solutions in the 0.5 mL syringes through 11 cm of tubing (0.01 cm diameter i.d.) at 32 rpm corresponds to a flow rate of 45 μL/s. Typically, a total of 12−16 μL of enzyme reaction was injected in a pulsed flow mode into the mass spectrometer at linear flow rates of approximately 3.9 m/s. All experiments were conducted under conditions to maintain turbulent flow (linear flow rates > 2.5 m/s) (*11*, *12*). To avoid problems which could occur with the buildup of pressure in driving the solutions in glass syringes through the narrow bore capillary, it was necessary to provide limits for speed and volume of solution delivery into the mass spectrometer. We did not experience problems with speeds of 256 rpm or lower and delivery volumes of 50 μL or less. Mass spectral scanning was begun just prior to the initiation of the pulse of enzyme reaction and an average of 6−9 scans for each individual pulse were collected for each mass spectra.

Mass Spectrometry. Mass spectrometry was performed using an ion trap mass spectrometer (Finnigan LCQ, Finnigan MAT, San Jose, CA) equipped with a Finnigan ESI source. All mass spectra were acquired using a signal-averaging protocol in centroid mode. Standard source conditions were as follows: capillary voltage (−4.1 kV); sheath gas (N_2), 90 psi; auxiliary gas (N_2), 10 units; heated capillary temperature, 240 °C. Standard ion trap conditions were as follows: ion injection time, 60 ms; full scan from 150−450 amu with 1 μscan resulting in a scan time of 180 ms.

Chemicals and Reagents. The S3P used in this experiment was a kind gift from Dr. Dan Studelska. The PEP was purchased from Aldrich Chemical Co. The EPSP was synthesized enzymatically from S3P and PEP with EPSP synthase as previously described (*2*). All the buffers and other reagents used in this study were of the highest commercial purity. The [1-^{13}C]PEP was purchased from Cambridge Isotopes (Andover, MA).

Enzymes. EPSP synthase was purified as previously described (*2*). Just prior to conducting rapid-mixing, pulsed-flow experiments, the enzyme was dialyzed into 10 mM ammonium acetate buffer, pH 7.8. The conditions for all rapid-mixing experiments were 10 mM ammonium acetate at pH 7.8 at 20 °C. All concentrations cited in the text are final concentrations after mixing.

RESULTS AND DISCUSSION

The enzyme 5-enolpyruvoyl-shikimate-3-phosphate (EPSP) synthase was chosen to demonstrate the utility of this technique. EPSP synthase is an enzyme found in plants and bacteria that is involved in the biosynthesis of a key precursor for aromatic amino acids. The enzymatic reaction for EPSP synthase involves the conversion of shikimate-3-phosphate (S3P) and phosphoenol pyruvate (PEP) to EPSP and inorganic phosphate (*2*). Mechanistic studies involving EPSP synthase have previously shown that the reaction proceeds through a tetrahedral intermediate formed transiently at the enzyme active site from a nucleophilic attack of the 5-OH of S3P on the sp^2 carbon at the C-2 position of PEP as illustrated in Figure 2 (*1*−*3*). These studies have also demonstrated that the EPSP synthase reaction is ordered with S3P binding to the enzyme first, followed by PEP.

The successful application of the rapid-mixing, pulsed-flow ESI methodology to EPSP synthase initially involved demonstrating the catalytic activity of the enzyme in a volatile buffer required for efficient electrospray ionization. It was found that 10 mM ammonium acetate at pH 7.8 served as an appropriate buffer for retaining maximal activity and was also suitable for MS studies. In pilot experiments to

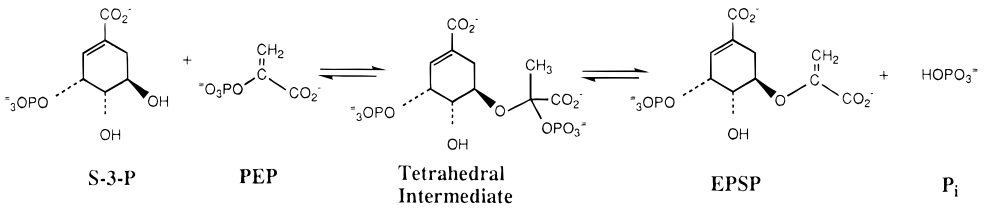
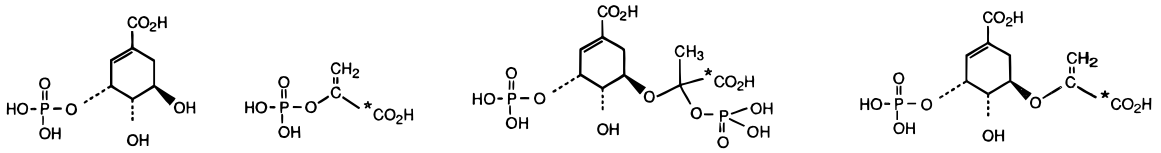


FIGURE 2: Reaction mechanism for EPSP synthase.

Table 1: Structures and Predicted MW for Substrates, Product, and Intermediate Using Negative Ion ESIMS[a]



	S-3-P	PEP	Tetrahedral Intermediate	EPSP
$[M - H]^-$	253	167 (335)[b]	421	323
$[M^* - H]^-$	No Change (253)	168 (337)[c]	422	324

[a] (*) ^{13}C-labeled. [b] Source induced dimer [2M − H]. [c] Source induced dimer [2M* − H].

examine the detection sensitivity of substrates (S3P and PEP) and product (EPSP), poor sensitivity was observed using positive-ion ESIMS. However, excellent sensitivity (~6 pmol) was realized when the ESIMS was carried out using ammonium acetate as a carrier combined with negative ion detection of molecular ions [M − H] for S3P, PEP, and EPSP. The structures and predicted molecular ions for EPSP synthase are shown in Table 1. In the rapid mix, pulsed-flow mode with fast scanning, the molecular ion sensitivity was still observed to be quite good [<36 pmol (12 μL of a 3 μM solution of substrate)].

Since the EPSP synthase reaction is ordered with S3P binding to the enzyme first, experiments to detect the tetrahedral intermediate would be conducted by rapidly mixing a solution of enzyme and S3P with a solution of PEP. Our previous rapid chemical quench studies examining the time course for the formation of the tetrahedral intermediate have shown that the intermediate can be observed at enzyme reaction times ranging from 3 to 100 ms (*2*). Under the conditions used in these studies, the concentration of the intermediate peaks around 20 ms and comprises approximately 20% of the radiolabel. At longer reaction times (>100 ms), complete conversion of limiting substrate to product occurs (*2*). If a limiting amount of enzyme and S3P is mixed with an excess of PEP for a reaction time of 20−30 ms, a distribution of substrates (S3P and PEP), tetrahedral intermediate, and product (EPSP) at the enzyme active site should be observed in the rapid mix, pulsed-flow ESIMS experiments with approximately 20% as tetrahedral intermediate.

Standard rapid chemical quench methodology requires that the enzyme and substrate solutions be mixed rapidly to maintain turbulent flow. Turbulent flow through a tube is predicted by the Reynolds number which is a dimensionless parameter dependent upon the flow rate, viscosity, and diameter of tubing (*11*, *12*). To insure proper mixing, it was imperative in our studies to carry out the mixing of solutions under conditions to promote turbulent flow and avoid problems associated with laminar flow (*11*). In practice, turbulent mixing requires a minimum linear flow rate of 2.5 m/s (*11*). The detection of the tetrahedral intermediate involved rapidly mixing a 6 μL aliquot of a preincubated solution of S3P (500 μM) and enzyme (250 μM) with a 6 μL aliquot of PEP (1000 μM) for a total reaction time of approximately 28 ms before introduction into the mass spectrometer. The mass spectra collected from this experiment are shown in Figure 3A. The molecular ions [M − H] are observed for the substrates S3P (*m/z* 253) and PEP (*m/z* 167), the product EPSP (*m/z* 323), as well as the putative tetrahedral intermediate (*m/z* 421). The intensity of the mass peak for the tetrahedral intermediate is consistent with previous kinetic studies which predict 15−30% (*2*). Control experiments in which either the enzyme-S3P solution or the PEP solution was substituted with buffer did not show the formation of the product, EPSP, or tetrahedral intermediate (spectra included as supporting information). Furthermore, mixing for extended periods of time (greater than 1 s) showed complete conversion of tetrahedral intermediate to the EPSP product as well as complete consumption of the limiting substrate (S3P) to form EPSP (spectra included as supporting information). Although the mass spectra appear to have chemical noise compared with the ESI spectra of proteins, this is in part due to the nature of electrospray in the low mass range (<500 amu). The relatively high noise in the spectra may also be due in part to the effect of the pulsed liquid flow to the mass spectrometer. Improvement on the quality of the pulsed-flow mass spectra may be achieved by introducing a sheath liquid for the electrospray and/or further modification of rapid-mixing interface. However, all the ions with significant signal intensity relative to background are identifiable and confirmed either as dimers and adduct ions related to the enzyme reaction or related to ammonium acetate buffer.

To further verify that the *m/z* 421 peak is due to the enzyme-catalyzed formation of the tetrahedral intermediate, the reaction was repeated using isotopically labeled [1-^{13}C]-PEP. Since the enzyme reaction involves a condensation between S3P and PEP, (Table 1), molecules containing a

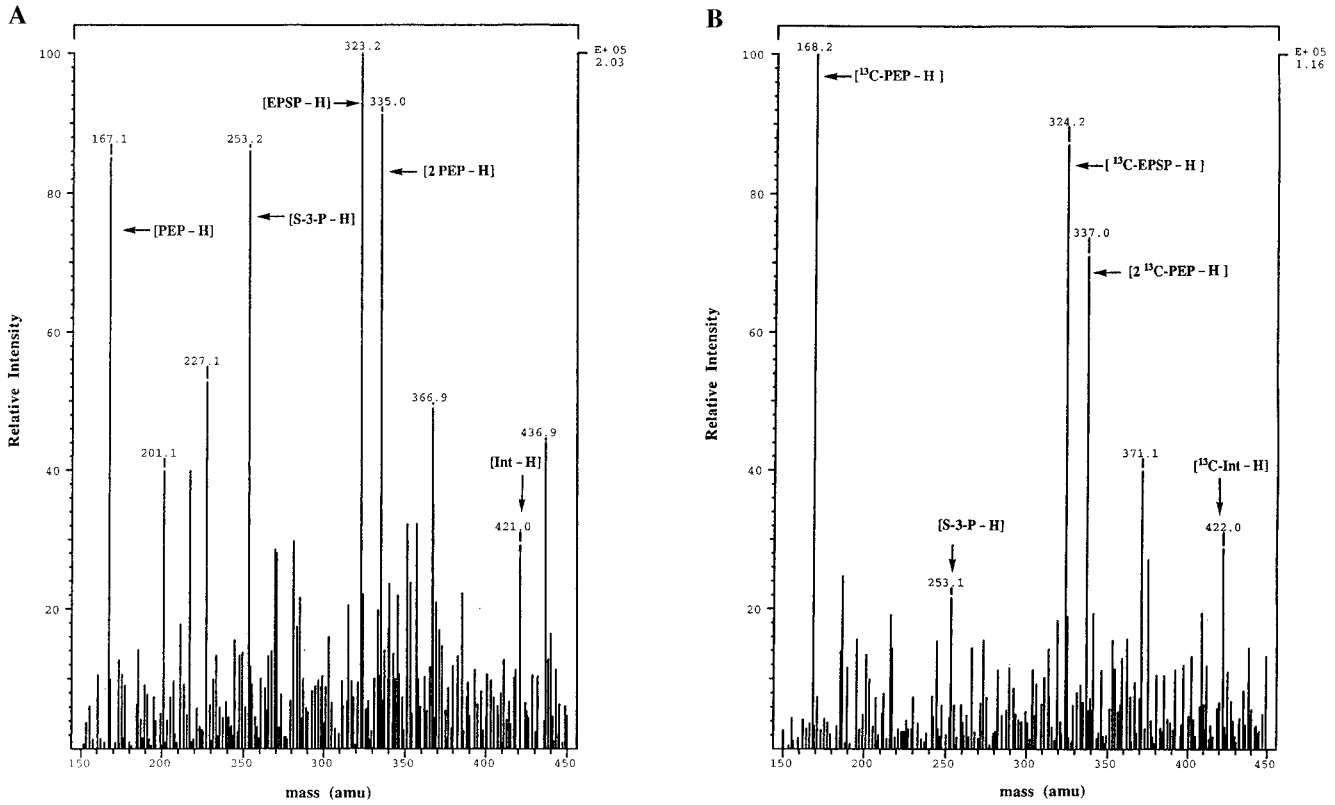


FIGURE 3: Detection of the tetrahedral intermediate in the EPSP synthase reaction. (A) Negative-ion electrospray ionization mass spectra of EPSP synthase reaction. A solution containing enzyme (250 μM) and S3P (500 μM) was rapidly mixed with PEP (1000 μM) for approximately 28 ms before quenching into the electrospray mass spectrometer. Arrows denote peaks for substrates (S3P and PEP), product (EPSP), and tetrahedral intermediate (Int). The mass spectra represent an average of six scans collected after an individual pulse of solution. (B) Negative-ion electrospray ionization mass spectra of EPSP synthase reaction using isotopically labeled PEP. A solution containing enzyme (250 μM) and S3P (500 μM) was rapidly mixed with [1-^{13}C]PEP (1000 μM) for approximately 28 ms before quenching into the electrospray mass spectrometer. Arrows denote peaks for substrates (S3P and PEP), product (EPSP), and tetrahedral intermediate (Int). The mass spectra represent an average of nine scans collected after an individual pulse of solution.

PEP moiety would have the ^{13}C-isotopic marker. Therefore, a shift of one additional atomic mass unit (amu) would be predicted for PEP and EPSP, as well as the tetrahedral intermediate but not for S3P (Table 1). Indeed, experimentally, the peaks corresponding to PEP (*m/z* 168), EPSP (*m/z* 324) and the tetrahedral intermediate (*m/z* 422) are all shifted by one additional amu while S3P (*m/z* 253) is unchanged (Figure 3B). Variation in peak intensity is noted in comparing panels A and B of Figure 3. The combination of these two experiments with unlabeled and [1-^{13}C]-labeled PEP establish definitively that the formation of the tetrahedral intermediate species is dependent upon the enzyme catalyzed reaction. Additional confirmation of the assignment of this peak as the tetrahedral intermediate was provided from the fragment ions generated during the collision-induced dissociation (CID) by a MS/MS experiment. Previous studies examining the chemical breakdown of the tetrahedral intermediate in solution have shown that one of the major products is EPSP resulting from loss of the phosphate attached to the tetrahedral center (*13*). The MS/MS experiment by daughter ion scan was carried out for the tetrahedral intermediate peak at *m/z* 421. Under these conditions, a peak at *m/z* 323 was observed which is most likely EPSP which may arise from loss of phosphate [M $-$ OPO_3H_3] (data not shown).

CONCLUSIONS

In summary, these ESIMS results demonstrate the utility of this novel rapid-mixing/electrospray MS technique for detecting transient enzyme reaction intermediates on the time scale of 20−50 ms in a well-characterized enzyme system such as EPSP synthase. In our preliminary studies, we have focused on the detection of intermediates rather than the potential use of the rapid mix, pulsed-flow ESI instrument in quantitative kinetic studies. However, we are in the process of further developing the technique using an optimal ESI interface and quantitative mass spectral detection to demonstrate the feasibility for defining the kinetics of chemical and enzyme reactions. We believe that this technique may hold general utility in monitoring dynamic biological processes such as enzyme reactions (*14*). This technique provides a means for observing transient enzyme intermediates and enzyme intermediates that are too chemically labile to be isolated with chemical quenching methods. Efforts are currently in progress to demonstrate the broader applicability of this technique to other enzyme systems and to the detection of covalent as well as noncovalently bound enzyme intermediates.

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BSF Grant 94-00371 to Dr. Timor Baasov whose enzyme system inspired the development of this methodology.

SUPPORTING INFORMATION AVAILABLE

Mass spectra of control reactions (2 pages). Ordering information is given on any current masthead page.

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