KINETIC ISOTOPE EFFECTS ON THE AROA REACTION: A Mechanistic

Investigation of the AroA Reaction

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KINETIC ISOTOPE EFFECTS ON THE ACID-CATALYZED HYDROLYSIS OF PHOSPHOENOL PYRUVATE AND THE AROA REACTION

By

GIRIJA DHEKNEY B.Sc, B. Ed

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Title: Kinetic Isotope Effects on the AroA Reaction: A Mechanistic Investigation of the AroA Reaction.

Author: Girija Dhekney B.Sc., (University of Waterloo), B. Ed. (Queen's University)

Supervisor: Dr. Paul J. Berti

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Abstract:

AroA catalyzes the synthesis of enolpyruvyl shikimate 3-phosphate (EPSP) from phosphoenolpyruvate (PEP) and shikimate 3-phosphate (S3P). It is part of the shikimate pathway which produces most aromatic compounds in plants, bacteria, and apicomplexa. The shikimate pathway is missing in mammals making AroA a potential antimicrobial target. It catalyzes a two step mechanism where the hydroxyl group of S3P adds across the double bond of PEP to form a tetrahedral intermediate (THI). Elimination of phosphate from the THI completes the reaction. In this study, kinetic isotope effects (KIEs) on the enzymatic reaction, as well as the non-enzymatic acid-catalyzed breakdown of PEP, were measured. A method for measuring KIEs by whole molecule mass spectrometry was developed. KIEs were determined using isotopologues of PEP. The KIEs measured on the enzymatic reaction using $[3.3-^{2}H_{2}]PEP$ was 0.985 ±0.003 and $[3-^{13}C]PEP$ was 0.97 ± 0.01. The KIE measured on the non-enzymatic reaction using $[3,3^{-2}H_2]PEP$ was 1.180 ± 0.009. Acid-catalyzed PEP breakdown proceeds through protonation at C3 to form an oxocarbenium ion intermediate. This is proposed to mimic the first step in the AroA-catalyzed reaction of S3P to PEP.

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For my parents and grandparents...

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List of Abbrevations

AroA	 enolpyruvyl shikimate-3-phosphate synthase
AroK	- shikimate kinase
DTT	- dithiothreitol
EDTA	- ethylenediamine tetraacetic acid
EPSP	- 5-enolpyruvyl shikimate-3- phosphate
IPTG	- isopropyl-β-D-thiogalactopyranoside
KIE	- kinetic isotope effect
NMR	- nuclear magnetic resonance
MG/AM	- Malachite Green + ammonium molybdate (for phosphate assays)
PEP	-phosphoenolpyruvate
PMSF	- phenylmethylsulfonyl fluoride
PPDK	- pyruvate phosphate dikinase
PPiase	- inorganic pyrophosphatase
Pi	- inorganic phosphate
S3P	- shikimate-3-phosphate
SDS-PAGE	- sodium dodecyl sulfate - polyacrylamide gel electrophoresis
THI	- tetrahedral intermediate
TS	- transition state
ZPE	- zero point energy

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1 INTRODUCTION

Resistance to drugs that were once thought to be permanent solutions to diseases such as tuberculosis and malaria has increased in the last few decades (1-3). As a result, the need to design new and more potent drugs has increased. Many of the drugs that are being designed are inhibitors of enzymes (4). They catalyze key reactions that allow bacteria to propagate and survive (5). Many of the inhibitors, either synthetic or naturally occurring, resemble either the substrate (6), product (7) or more recently, the transition state structure of the enzyme catalyzed reaction (8).

AroA and MurA are enzymes that have been studied extensively due to their potential as targets for new antimicrobial agents (9). AroA, which is the focus of this project, is part of the shikimate pathway that produces aromatic amino acids and most aromatic compounds in bacteria, plants and apicomplexa (10, 11). The shikimate pathway is missing in mammals (10), which makes AroA a potential target for antibiotics.

1.1 AroA, a Carboxyvinyl Transferase

AroA is one of only two enzymes that can catalyze a carboxyvinyl transfer reaction (*12*). It catalyzes the reaction between shikimate 3-phosphate (S3P) and phosphoenolpyruvate (PEP) to produce 5-enolpyruvyl

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shikimate-3-phosphate (EPSP) and inorganic phosphate (P_i) (*12-16*) (Fig.1). The other enzyme that catalyzes a carboxyvinyl transfer reaction is MurA (Fig. 2).



Figure 1: AroA-catalyzed reaction.



Figure 2: MurA-catalyzed reaction.

1.2 Structure and General Mechanism of AroA

AroA is a 46 kDa protein and is comprised of a single polypeptide chain containing 462 amino acids (*15, 16*). The single polypeptide chain folds into two similar domains which come together to form the active site upon binding S3P. Each of the two domains is an inside-out α/β barrel which is made up of a 3-fold repeating unit of $\beta\alpha\beta\alpha\beta\beta$ motif (*15*). The overall structure and the induced fit mechanism, meaning that the enzymes undergo a conformational change upon binding of the substrates, are common to both AroA and MurA (*15-18*).

The AroA reaction follows a two-step mechanism and proceeds via an addition-elimination process (*9, 15, 16, 19-21*). During the addition step, the C2 carbon undergoes nucleophilic attack from 5'OH of S3P (Fig. 3). C3 of PEP is protonated by an amino acid in the active site (*9*). This gives the tetrahedral intermediate (THI). During the elimination step, C3 of PEP is deprotonated to form the double bond between C2 and C3 while eliminating the phosphate group of the THI to give the products EPSP and P_i.



Figure 3: Mechanism of the AroA reaction.

1.3 AroA as a Drug Target

Glyphosate is a herbicide that inhibits enolpyruvylshikimate 3-phosphate synthase (EPSPS), which is a plant orthologue of AroA (22). It also inhibits AroA, but it is not an antibacterial. Glyphosate is unable to inhibit MurA, which makes it an inhibitor specific to AroA (22). It has been shown to slow growth in bacteria and apicomplexa; however, it is not an antibiotic (23, 24). Therefore a better inhibitor is needed.

Glyphosate resembles PEP and has been shown to bind to the same site as PEP on AroA (10, 22, 25) (Fig. 4). The K_d for glyphosate is 0.09 μ M (26). It is the active ingredient in the commericial herbicide RoundUpTM (27).



Glyphosate

Figure 4: Structure of glyphosate and protonated PEP showing resemblance to one another.

Recent experiments involving site directed mutagenesis (28), x-ray crystallography (29) and NMR (30-32), and studies involving enzyme kinetics have furthered our knowledge of the mechanism of this enzyme (9, 10, 14, 19, 20, 33-35). They have given a new insight into the residues involved in the catalysis of S3P and PEP, the stereochemical course of the reaction, and brought us one step closer to finding a better inhibitor of AroA.

1.4 Detailed Mechanism of AroA

It was shown previously by Anderson et al. that the AroA reaction follows a two step mechanism: addition followed by elimination, and that it proceeds via a tetrahedral intermediate (*13*, *36*). Anderson et al. also determined that the reaction is fully reversible at each step. Exogenously added THI partitions in the forward and reverse directions in a ratio of 3:1 (*13*).

1.5 Controversy Over the Stereochemistry of the AroA Reaction

To further study the mechanism of the AroA-catalyzed reaction, the stereochemistry of the reaction becomes important. The reaction could proceed through a number of stereospecific pathways. First, the addition of the proton could occur on the *si*-face or the *re*-face of PEP. Second, the addition of the S3P hydroxyl could occur on the same face (syn-addition) or the opposite face (anti-addition). Similarly, the proton could be extracted from either face in the elimination step, with syn or anti-departure of the phosphate. The relative stereochemistry is also important. It can be the same in each step (syn/syn or anti/anti), or opposite in each step (anti/syn or syn/anti).

Walsh and co-workers determined that the proton addition is on the *si* face of PEP. They did this by trapping fluorinated THI using (E)-and (Z)-fluoro-PEP and determining the configuration of the chiral fluoromethyl group formed when the reaction was run in D_2O (*37*). It had previously been established that the

addition and the elimination steps of the AroA reaction proceed with opposite stereochemistry (*38*). This was shown by the fact that the stereochemistry of the double bond of PEP is retained in EPSP by using stereospecifically isotope-substituted PEP (*19, 20*). However, the exact stereochemistry of the reaction still remained undetermined.

An. et al. determined that the reaction undergoes an *anti* addition followed by *syn* elimination. They came to this conclusion by synthesizing (R)-THI and (S)-THI and then reacting them with AroA. They found that (R)-THI was inert to AroA while (S)-THI reacted to produce EPSP, PEP and S3P. The fact that only (S)-THI was catalyzed, along with Walsh's results, demonstrates that the initial step of the reaction proceeds as an *anti* addition step (19). Mizyed et al. proposed acid/base catalytic residues that are consistent with the stereochemistry, however Eschenburg et al. disagreed with this proposal, based on crystal structures of mutant AroA and MurA with THI bound (15). All the available evidence indicates that AroA and MurA have identical stereochemistries of reaction.

1.6 Controversy on the Amino Acids Required for the AroA Reaction

Putative catalytic residues were identified previously from E•S and E•I co-crystal structures (*9, 28*). Previous mutagenesis experiments were able to identify amino acids as essential or not essential depending on whether enzymatic activity was lost upon mutagenesis. However, these experiments

could not distinguish whether a particular amino acid was crucial in the addition step, as distinct from the elimination step. Recently, our lab approached this problem by using partitioning analysis of purified AroA [1-¹⁴C]THI with AroA (9). Fourteen amino acids which were within 5 Å of a reactive atom in the AroA•THI model were mutated and a partitioning factor, f(PEP), was measured and compared to f(PEP) of wild type AroA. The partitioning factor, f(PEP), was calculated using equation (1).

$$f(\mathsf{PEP}) = \frac{[\mathsf{PEP}]}{[\mathsf{pyruvate}] + [\mathsf{PEP}]}$$
(1)

A partitioning factor of 1 would indicate no formation of EPSP and the elimination step of the reaction was affected by the mutation. A partitioning factor of 0 would indicate that the sole product of THI breakdown was EPSP and that the mutation affected the addition step. The partitioning factor for wild type AroA was 0.25.

Our lab also proposed that Lys22 acts as the general base catalyst during the addition step deprotonating the 5'OH of S3P. It then acts as the general acid which protonates either the phosphate group or 5'OH of S3P to promote departure and produce EPSP or S3P in the forward or reverse reactions, respectively (Fig. 3). It was proposed that Glu341 acts as the general acid catalyst which protonates C3 of PEP during the addition step, and then is a general base to deprotonate C3 of the THI during the elimination step. In short,

both Lys22 and Glu341 act as both acid and base catalysts in both steps of the AroA reaction. Evidence for this included: (i) a very small effect on f(PEP) with the K22A and K22R mutants, (ii) a very small effect on f(PEP) with the E341A and E341Q mutants (iii) invariance of f(PEP) at high pH, and (iv) proper location in the crystal structures (9).

The very small effect on *f*(PEP) can be attributed to the fact that Lys22 acts as both acid and base catalyst and therefore both addition and elimination steps of the reaction will be affected by mutation. Further evidence that only a single amino acid is involved stems from the fact that as the pH increases, the protonation state of the amino acid changes and both the forward and the reverse reactions are affected equally. Lys22 is correctly situated to catalyze the reaction in the proposed manner. According to the crystal structures of AroA•S3P•glyphosate, AroA•S3P•phosphate•formate and AroA•THI structures, the N atom of the lysine side chain was in contact with at least one of the oxygen atoms of either the phosphate moiety and/or the carboxylate oxygen atom of glyphosate or THI. Lys22 is therefore correctly situated to donate a proton to 5'OH of S3P or the phosphate group of THI. Glu341 was proposed to act as general acid and general base catalyst in the overall AroA reaction. This proposal is supported by the following evidence: (i) it is correctly located to catalyze this reaction, and (ii) no significant changes in the *f*(PEP) occur in the mutants E341A and E341Q (9).

Recently, residue Cys115 of MurA was identified as the amino acid that acts as a general acid/base catalyst to protonate and deprotonate C3 of PEP (*20*). When the structures of AroA and MurA were superimposed, the sulfur atom of Cys115 was within 0.8Å of the carbon of the carboxylate group of Glu341 side chain (9). Aspartate replaces Cys115 in 9 of the 48 known MurA sequences. This shows that Glu341 is properly situated and that the carboxylate groups are indeed functional and can catalyze the reaction by the proposed mechanism (*9*). The second line of evidence comes from the fact that no significant changes were seen in the *f*(PEP) with the E341A and E341Q mutations.

The phosphate group of the THI has been proposed by Bartlett and co-workers to catalyze its own elimination from THI to give EPSP (19). However if this were true, then another amino acid would have to act as the general base to catalyze the departure of S3P from THI in the reverse reaction. In this case, a shift in f(PEP) would have been observed with E341A and E341Q. Since this was not observed, the evidence does not support Glu341 as the other amino acid which acts as the general acid/base in the AroA reaction (9).

On the other hand, they suggest that Glu341 and Asp313 are both involved in the addition step of the reaction and the departure of the phosphate group is through intramolecular general acid catalysis of phosphate group elimination of THI. However, they do not comment on the departure of S3P in the reverse reaction. They explain this by referring to the exact stereochemical course of the

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enzymatic reaction. They verified the proton is added to the *si* face of the PEP double bond. They concluded that since Glu341 is positioned above the *si*-face of the double bond and opposite the 5'-OH group of S3P; it serves as a proton donor during the addition step, donating the proton to C3 of PEP. Asp313 is located adjacent to the 5'-OH group of S3P, and serves as proton acceptor of the 5'-OH proton during the nucleophilic attack of the 5'-OH of S3P on C2 of PEP (*19*).

They disagree with our lab's proposal that Glu341 could serve as a proton acceptor of the THI. They rationalize this idea by pointing out that the carboxylate group of Glu341 is oriented 90° to the C-O bond that must be broken to release the phosphate group. They claim that it would be almost impossible for Glu341 to reorient itself for the proper stereochemistry of the reaction which they propose occurs via *syn*-elimination of phosphate. They suggest that the phosphate group is self-eliminated since it is perfectly positioned for *syn*-elimination (*19*).

Eschenburg et al. recently solved the crystal structures of the D313A mutants of MurA and AroA with the THI bound (*15*). They proposed yet another pair of amino acids which are involved in the AroA reaction. They agreed with our lab that Lys22 acts as the general base catalyst during the addition step of the reaction, but suggested that Asp313 is the key catalytic residue during the elimination step (*9, 15, 19*). The evidence they gave to supported this theory was as follows: (i) the enzymatic reaction is halted after the addition step in the

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D313A mutant of AroA. They suggest that the aspartate side chain is therefore crucial for the elimination step, and therefore cannot act as a proton acceptor as suggested by An et al (*15, 19*). Mizyed et al. agree that Asp313 is properly situated for proton abstraction from the methyl group of the THI but would not allow for *syn*-elimination as suggested by Mizyed et al. as well as An. et al (*9, 15*). Eschenburg et al. agree that the stereochemistry would follow an *anti*-elimination, but propose that aspartate is the key residue in the elimination, since the reaction was halted in the D313A mutant after the addition step (*15*).

Based on their crystal structure of AroA•THI, Eschenburg et al. proposed that the reaction proceeds via a *syn* addition on the *re* face of PEP. This contradicts the previous work by Walsh and co-workers described above (Section 1.5). Eschenburg et al. agreed that the THI has an (*S*) configuration. They conclude Asp313 is the key active site residue responsible for the deprotonation of C3. It is on the *re* face of PEP and the phosphate leaving group is on the *si* face of PEP. Thus the elimination reaction is an *anti*- elimination. As a number of experiments have shown that the addition and elimination steps proceed with opposite stereochemistries they conclude that the addition step would have to be *syn* (9).

1.7 Non-enzymatic Breakdown of the Tetrahedral Intermediate

Byczynski et al. recently examined the non-enzymatic breakdown of the THI of AroA to gain better understanding of the corresponding enzymatic reaction

(12). By studying the rates of the reaction and product distribution of the THI breakdown at different pHs, they were able to provide evidence for the following: (i) that the reaction proceeds via a C-O rather than P-O bond cleavage, (ii) formation of an oxocarbenium:phosphate ion pair complex, and (iii) general acid catalysis (12). They showed C-O bond cleavage by degrading MurA THI in the presence of 50% aqueous methanol where, if a P-O bond cleavage were to take place, methanol would react with the metaphosphate intermediate to form monomethyl phosphate. Since there was no monomethyl phosphate formed, it was concluded that the reaction undergoes a C-O bond cleavage (12).





They further stated that an oxocarbenium-phosphate ion complex is formed immediately after C-O bond cleavage (Fig. 5). This conclusion was based on the observation that under acidic conditions without an ion pair complex, the oxocarbenium ion would undergo a rapid water addition to yield pyruvate only, since the oxocarbenium ion is a highly electrophilic ion. With increasing pH however, the negative charge on the phosphate group increased, stabilizing the ion pair. The extended lifetime of the ion pair allowed other reactions in addition to water attack, namely deprotonation to give an overall phosphate elimination, as well as intramolecular attack from the adjacent hydroxyl group of the alcohol to yield the ketal product (39).

The increased yield of both these products with increasing pH can be explained with the protonation state of the phosphate group. A dianionic phosphate is better able to stabilize the oxocarbenium ion than a monoanionic phosphate. Evidence that THI breakdown is general acid-catalyzed, stems from the observed solvent deuterium KIE. The solvent deuterium KIE at pH 2.0 was 1.3 ± 0.4 which is typical of general acid catalysis of various acetal hydrolyses (*12*).

1.8 Non-enzymatic Hydrolysis of PEP

Evidence shows that both AroA and MurA catalyze formation of oxocarbenium ion intermediates or oxocarbenium ion-like transition states. It has been shown that PEP exchanges its protons during the AroA-catalyzed reaction

(40, 41). This is only possible by formation of an oxocarbenium ion intermediate. Glyphosate, an inhibitor of AroA, bears a positive charge near where one would be expected for a cationic intermediate, which supports the postulated formation of an oxocarbenium or an oxocarbenium ion-like intermediate. Also, formation of S3P ketal in the AroA reaction and during the non-enzymatic breakdown of THI can be explained by formation of an oxocarbenium ion or oxocarbenium ion pair complex (*12*).

The AroA-catalyzed reaction proceeds via protonation of C3, then THI formation, followed by C-O bond cleavage to eliminate phosphate. To compare the transition state of the non-enzymatic *versus* the enzymatic reactions, KIEs have to be determined under conditions where the C3 of PEP is protonated and the double bond is broken to form a carbocation. Wolfenden and co-workers showed that under acidic conditions with pHs as low as 0, PEP undergoes P-O bond cleavage which is not relevant to the AroA reaction (*42*). However, based on the pH-dependence of the breakdown of enolpyruvyl group, it would be expected that C3 protonation in PEP to form an oxocarbenium ion would dominate at very low pH (*43*). Therefore, at pH lower than 0 PEP hydrolysis should occur predominantly through C-O bond cleavage. It was also shown by Benkovic and Schray that the non-enzymatic hydrolysis of PEP occurs only at elevated temperatures (*44*).



Figure 6: Breakdown of PEP under acidic conditions

1.9 Kinetics and the Reaction Mechanism

Kinetics is a tool used by chemists and biochemists to study the mechanism of a reaction (45). Kinetics describes the rates of reactions under varying conditions, and it tells us how the rate of the reaction is dependent on the concentration of reactants. A reaction mechanism is a sequence of elementary steps, unimolecular or bimolecular, which can be invoked to account for a chemical change. The dependency of concentration on rate, reflects the number of molecules that must be present for a particular step to occur. In a unimolecular step, the reaction rate depends only on the amount of reactant present. That is, it is a first order reaction. In a bimolecular step, the reaction rate depends on both reactant concentrations and is therefore second order in those reactants (46). At any given temperature, any molecule will have kinetic and potential energy. The kinetic energy is in the form of vibrational, rotational, translational and electronic energy. The translational energies of a molecule will be dependent on the molecular mass, the rotational energy will depend on the principal moments of inertia, while the vibrational energy will depend on the normal vibration frequencies and the electronic energy levels (47).

The rate of the reaction, and factors that influence the rate such as pH,

temperature, catalysts and isotopic substitutions can tell us a great deal about the mechanism of the reaction.

1.10 Reaction Co-ordinate and the Transition State



REACTION CO-ORDINATE

Figure 7: Diagram of a reaction co-ordinate plotted against free energy showing reactants, products and TS along the reaction co-ordinate.

Energy is plotted against the reaction co-ordinate (Fig. 7). The energies of the reactant and product are local minima, with all bond lengths and bond angles fully optimized. The molecular structure corresponding to the maximum energy

between the reactant state and product state along the reaction co-ordinate is

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known as the transition state (46). According to transition state theory, the transition state is considered to be just like a stable molecule except for motion of atoms along the reaction co-ordinate (47). That is, all bond lengths and angles are fully optimized, except for motion in the reaction co-ordinate, where the energy is at a maximum. The reaction co-ordinate is the vibrational mode along which the decomposition of the transition state occurs. The theorem of Murrell and Laidler states that a transition state must possess one and only one unstable vibrational mode, the reaction co-ordinate (47). If all vibrational modes are stable, then the molecule is an intermediate, a reactant or a product. A simple treatment of transition theory assumes that the reactants are in rapid equilibrium with the transition state (47). Thus, the elementary step

A + B \xrightarrow{k} P is actually treated as,

A + B
$$\xleftarrow{K^{\ddagger}} [AB]^{\ddagger} \xrightarrow{k^{\ddagger}} P$$

The rate law for the elementary step then becomes $k = K^{\dagger}k^{\dagger}[A][B]$, where *k* is the rate constant between reactants and products, K^{\dagger} is the equilibrium constant between reactants and the transition state and k^{\dagger} is the rate constant for transition state breakdown.

The relationship between reaction rate and activation energy is shown in equation (2):

$$k = \left(\frac{k_{B}T}{h}\right)e^{\left(\frac{-\Delta G^{\ddagger}}{RT}\right)}$$
(2),

where k_B is the Boltzman constant, *T* is the temperature in Kelvin, *h* is Planck's constant, ΔG^{\ddagger} is the difference in Gibbs free energy between reactants and the transition state and *R* is the gas constant. Transition state theory relates kinetic rates to the thermodynamic properties of reactants and the transition state (46).

1.11 What are KIEs?

A kinetic isotope effect (KIE) reflects the change in reaction rate upon isotopic substitution at a given position. By definition KIE = $^{light}k/$ $^{heavy}k$, where $^{light}k$ and $^{heavy}k$ are the rate constants for any elementary step passing through a transition state, with $^{light}k$ corresponding to the rate of the lighter isotopologue (*e.g.* ¹H or ¹²C) and $^{heavy}k$ corresponding to the rate of the heavier isotopologue (*e.g.* ²H or ¹³C) (47). KIEs reflect the vibrational energy of the molecule at the transition state. A vibrational environment can be either loose or tight. Atoms in a molecule can be imagined to be like masses held together by springs. If the strength of the spring becomes weaker, that is, if the bond between the two atoms is longer or broken at the transition state, then the atoms are said to be in a looser vibrational environment than in the reactant state. If the spring becomes stronger, that is, if the bond becomes shorter and stronger at the transition state, the atoms are said to be in a tighter vibrational environment.

KIEs provide information on whether bonds are being broken or made at the transition state (47). For example, if the bond containing the labeled atom is broken, or if the bond between the two is getting weaker, the atom is said to be in a looser vibrational environment, and the KIE will be normal, *i.e*; > 1. If the vibrational environment of the atoms is tighter, the KIE will be inverse, *i.e*; < 1 (Fig. 8) (47).



Figure 8: Examples of normal and inverse KIEs (8).

1.12 What gives rise to KIEs?

Atoms connected together by a bond can be treated as a harmonic oscillator where, if a bond is stretched or compressed, there exists a corresponding force that tries to restore the bond to its equilibrium position. The stretching and compression of the bonds constitutes the vibrational frequency of the bonds and is dependent on the masses and strength of the bond. Atoms cannot be at rest even at 0K. They continue to vibrate and have energy known as the zero point energy (ZPE). Note that at the temperatures used in this study, bonds remain predominantly in their lowest vibrational state, where ZPE is equal to their vibrational energy. However, during a chemical reaction if the structure changes between the reactant and the TS, the ZPE will also change. This will affect the overall energy of reactant and transition state molecules. As the ZPE is dependent on the mass of the atoms, the energies of the reactants and transition states also depend on the mass of the atoms. The energy requirements to reach the transition state for the two isotopologues will be different (48). For example, the higher the frequency of the bond, the easier it is to break the bond. The ΔG^{\ddagger} required to break the bond is less for the bond with higher vibrational zero point energy at the ground state (Fig. 4). The rates of the reaction are dependent on the difference in Gibbs free energy between the reactant state and the transition state by equation (2) (46). Therefore the difference in ΔG^{\ddagger} suggests there will be a difference in the rates of reaction between the molecule and its isotopologue.

1.13 What do KIEs tell us?

The ZPE is influenced by the masses of the atoms and the strength of the bond that holds the two atoms together (*47*). The vibrational energy of a bond is

inversely proportional to μ , that is E $\propto 1/\mu$, where μ is the reduced mass and is given by equation (3):

$$\mu = \frac{(m_1 m_2)}{m_1 + m_2} \tag{3},$$

where m_1 and m_2 are masses of atoms held together by a bond. So, if we compare a C-H *versus* a C-D bond, the vibrational ZPE for a C-H bond is higher than a C-D bond.

If two atoms are imagined to be held together by a spring, increasing the strength of the bond will also increase its frequency. Thus, the vibrational ZPE is also dependent on the strength of the bond. The stronger bond is, the higher its vibrational ZPE (Fig.9).



Figure 9: Differences in vibrational zero point energies between reactant state and transition state for a normal KIE (right) and an inverse KIE (left). If the bond bearing the isotopic label becomes weaker at the transition state, the energy difference between C-H and C-D becomes smaller, and the activation energy for C-H becomes smaller than C-D. This causes a normal KIE. Conversely if the bond bearing the isotopic label becomes stronger at the transition state, the energy difference between C-H and C-D becomes label becomes compared the isotopic label becomes stronger at the transition state, the energy difference between C-H and C-D becomes larger and the activation energy for C-H becomes larger than C-D, giving an inverse isotope effect.

As described earlier, the rates of reaction are dependent on the energy of the molecule at the reactant state which depends on the molecular structure of the reactant molecules (*47*). When one of the atoms in the molecule is substituted with its heavier isotope, a difference in rate is observed due to the difference in ZPE of reactants (*47*). The change in rate of the reaction can tell us a great deal about the reaction mechanism. The size of the KIEs is determined by how much the structure changes at the transition state. By determining KIEs at many sites in the reactant molecule, it is possible to determine the structure of the transition state. KIEs on the atoms involved in actual chemistry, where bonds are either made or broken, are primary isotope effects, while those not involved in the actual chemistry are secondary isotope effects (*47*).

1.14 Enzymes and their Transition States

Enzymes are proteins that act as catalysts. For the reaction $A \rightarrow P$, A goes through a transition state (49). Enzymes catalyze the reaction by stabilizing the transition state or forming a different transition state having a lower energy of activation (50). Enzymes lower activation energy for the reactions by binding to, and stabilizing the transition states. However, the TS to which the enzyme binds is not necessarily the same as for the non-enzymatic reaction: they can also lower the activation energy by changing the TS structure (50). Enzymes can do this because the environment of the active site is different from aqueous medium (50). The activation energy is lowered by the enzymes in many different ways. One is to bind the substrates to different parts of the enzyme, causing a conformational change in the enzyme which brings the substrates together in the proper orientation to go forward with the reaction. Also, the pKa of each amino acid can be fine tuned so that each side chain is in the correct protonation state. This can allow for simultaneous protonation and deprotonation, which is highly improbable in solution (50).

1.15 Enzyme Kinetics

Enzyme kinetics is the same as any chemical reaction except for the phenomenon of enzyme saturation, which does not exist in non-catalyzed chemical reactions (*45*). At low substrate concentrations, the rate increase is proportional to substrate concentration. However, at high substrate concentration the rate stabilizes and becomes independent of substrate concentration due to saturation of the enzyme with substrate.

A number of terms and equations are used to describe enzyme kinetics and they will be briefly reviewed here. Enzyme kinetics is described, in the simplest case where there is only one substrate, by the Michaelis-Menten equation. The Michaelis-Menten equation is equation (4):

$$A + E \xrightarrow{K^{\ddagger}} [AE]^{\ddagger} \xrightarrow{k^{\ddagger}} P + E$$

$$v_{0} = \frac{k_{cur}[E]_{0}[A]_{0}}{[A]_{0} + K_{M}} \qquad (4),$$

where v_0 is the initial rate, k_{cat} is the maximum rate, [E]₀ is the initial enzyme concentration, [A]₀ is the initial substrate concentration and K_M is the substrate concentration at which the rate is one-half k_{cat} . This equation is true only under steady state assumption which states that the concentration of the Michaelis complex E·A, stays constant, that [A]₀ >> [E], and k₂ is much slower than k₋₁. The other steady state parameters often used to describe enzyme kinetics are k_{cat} and k_{cat}/K_{M} . The constant k_{cat} is known as the enzyme turnover number and is the number of product molecules made per active site per second. The constant k_{cat}/K_{M} is known as the enzyme specificity constant. It reflects the activation energy between the free enzyme and free substrate in solution and the transition state of the first irreversible step of the reaction.

1.16 Enzymes and Transition States of a Reaction

As stated earlier, enzymes catalyze reactions by binding to the transition state. Enzymes generally bind to substrates and products with equilibrium dissociation constants, K_d s, in the 10⁻³ to 10⁻⁶ M range. Tighter binding of the product or the reactant would interfere with product release. Enzymes can bind to transition states with K_d s as low as 10⁻²⁴M (*51*).

Competitive inhibitors bind to the enzyme and block the enzyme's active site thus preventing it from catalyzing the reaction. Since enzymes bind to TS much more tightly than either the reactants or products, analogues of transition states are more likely to be more potent drugs than the reactant or product analogues. However, the question of the antibiotic resistance still remains. TS analogues do however make it difficult for bacteria to develop resistance since resistance to TS analogues must arise through specificity against remote parts of the inhibitor molecule because the enzyme must retain its ability to bind to the transition state.
1.17 Measurement of KIEs

TS structures last less than a single bond vibration, $\approx 10^{-13}$ s. Therefore, TSs cannot be isolated or analyzed using conventional analytical techniques such as NMR or X-ray crystallography to determine their structures. Measuring KIEs provides the most direct way to deduce TS structure in atomic detail.

There are many ways to determine KIEs. They include isotope ratio mass spectrometry, scintillation counting, whole molecule mass spectrometry, and NMR (*50, 52-54*). The advantages and disadvantages of each technique are briefly discussed in this section.

Of all the techniques used to measure kinetic isotope effects, isotope ratio mass spectrometry (IRMS) gives the most precise and accurate measurements. The precision of IRMS technique is 50 times greater than that of any other techniques. However, this technique is limited to small gaseous molecules such as N₂, CO₂, and Cl₂. The majority of kinetic isotope effects are measured using scintillation counting. The advantage of measuring KIEs by using radioactivity is that one is not limited to any size or type of molecule. Many radioisotopes exist and can be used to label molecules. Molecules need remote labeling and for such molecules, depending on the isotope, the lifetime of the synthesized molecule could be limiting. Also, synthesis of labeled molecules can be very expensive.

To avoid the costs and hazards associated with radioactivity, whole molecule mass spectrometry can be used to measure KIEs (52). It is a faster method than KIEs determined by radioactivity. The method is only applicable if ionization can occur without fragmenting the molecule because kinetic isotope effects on the fragmentation reaction are possible.

IRMS, radioactivity and whole molecule mass spectrometry all require synthesis of labeled substrates. Recently, a method was developed to measure KIEs on natural abundance isotopes using NMR (*54*). In order to measure KIEs on natural abundance isotopes the extent of reaction is taken to 90% completion and KIEs are measured by examining the isotope ratios in the residual substrate. The drawback to this approach is that a lot of material is needed, at least 30 to 50 mmoles (*54*).

1.18 Kinetically Significant Steps

When determining KIEs, it is important to know which step of the mechanism is reflected in the KIEs (Fig. 10). For KIEs on k_{cat} , the rate-limiting step(s) is the kinetically significant step, while for KIEs on k_{cat}/K_m , the kinetically significant step is the first irreversible step of the reaction. The rate-limiting step can also be the first irreversible step for a reaction. However, this is not always the case. The rate-limiting step is the step which has the greatest energy difference between either an intermediate or reactant and the transition state for that step. The first irreversible step is the step which has its transition state at the

highest overall energy point in the reaction co-ordinate. It is possible for a reaction to have two (or more) partially rate-limiting or partially irreversible steps (50).



Figure 10: Reaction pathways depicting the difference between first irreversible step, first rate-limiting step and partially irreversible step.

1.19 Competitive vs Non-competitive Methods of Measuring KIEs

KIEs can be measured by either competitive or non-competitive methods. Non-competitive methods to measure KIEs requires that the reaction with labeled and unlabeled substrate be done separately (47). Rate constants of each isotopologue are determined separately in order to give the KIEs. They reflect KIEs on the rate-limiting step of the reaction for non-enzymatic reactions. The advantage of non-competitive isotope effects with enzymatic reactions is that it is possible to measure, in theory, KIEs on every single kinetic parameter of a given reaction; that is, k_{cat} , k_{cat}/K_m , and K_m (50). However, non-competitive isotope effects are generally not used because the measurement of rate constants is not

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accurate enough for transition state analysis (*47*). Pipetting errors and other instrumental errors ensure that the errors in the KIEs will, usually, be too large for TS analysis. These sources of error are avoided when measuring competitive KIEs.

Competitive KIEs are measured by mixing labeled and unlabeled substrate in a single reaction mixture. If there is a KIE, there will be a change in isotopic ratios in the residual substrate or in the product after the reaction is taken part way to completion. Competitive isotope effects arise due to the difference in rate constants on steps up to and including the first irreversible step of the reaction. Competitive KIEs for an enzyme-catalyzed reaction are KIEs on k_{cat}/K_{m} , the specificity constant. It reflects the energetic relationship between free enzyme and substrate and the transition state of the first irreversible step of the reaction (50).

1.20 Partioning Analysis of the Enzymatic Breakdown of the Tetrahedral intermediate

Both the addition and elimination steps of the AroA reaction are partially irreversible. Initial partitioning analysis of the tetrahedral intermediate showed that at pH greater than 6, f(PEP) = 0.25. That is, the reaction went ³/₄ in the forward direction and ¹/₄ in the reverse direction. It was reported previously that at pH < 6, the AroA reaction partitions completely in the forward direction, making the addition step the first irreversible step (9, 14). However, more recently,

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experiments showed that this was partially a chromatographic artifact and that partitioning is not completely in the forward direction (Fuzhong Zhang, personal communication). Therefore KIEs measured at pH < 6 would reflect KIEs on both the addition and elimination steps. Several KIEs on the AroA reaction had already been measured at this point. The focus of this project was then shifted to the acid-catalyzed breakdown of PEP which is a mimic for part of the AroA reaction.

1.21 Objective of this Project

The objective of this project was to determine KIEs on the enzymatic and non-enzymatic steps of the AroA reaction. These KIEs can be used to determine the transition state structure whose analogs can be used as inhibitors of AroA. Such inhibitors could be lead compounds in the development of new antibiotics. Crystal structures of inhibitors that closely resemble the transition state complexed with the enzyme can be viewed as a snapshot of the enzyme at the transition state. This information could potentially help resolve the question of which amino acids are involved in catalysis. KIEs also help determine the mechanism of the AroA reaction. The method used for determining KIEs was by whole molecule mass spectrometry and one of the goals was to optimize experimental conditions so that KIEs could be measured precisely and accurately.

2 MATERIALS AND METHODS

2.1 Purification of Enzymes

2.1.1 AroA Expression and Purification

E. coli AroA, His₆-tagged at the C-terminus (AroAH₆), was expressed and purified as described by Mizyed et al. (9). Briefly, AroAH₆ which had been cloned into a pET23a vector was transformed into BL21 star (DE3) E. coli cells. A colony was selected from an {LB agar + 30 µg/mL kanamycin} plate and grown overnight in LB broth + 30 µg/mL kanamycin. Cultures (6 × 1 L) were inoculated with 5 mL each of overnight culture, and were grown at 37 $^{\circ}$ C until OD₆₀₀ reached 0.6 to 0.7. They were then were induced with 1 mM IPTG (isopropyI-B-D-thiogalactopyranoside). Cultures were allowed to grow for 4 hours at 37 °C while shaking at 150 rpm. Cells were collected by centrifugation at 10,000 × g for 10 min at 4 °C. Cells were resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 20 mM imidazole, 0.3 M NaCl, 1 mM PMSF and 100 mM benzamidine-HCI. DNase and RNase were added to the resuspended solution before lysing the cells by 3 passes through a French Press at 10,000 psi. Cell debris was removed by centrifugation at 25,000 \times g at 4 $^{\circ}$ C. AroAH₆ was purified by affinity chromatography using a 20 mL chelating column which was charged with Ni²⁺SO₄²⁻. AroAH₆ was loaded using lysis buffer and eluted using elution buffer containing 0.3 M NaCl, 500 mM imidazole, 50 mM Tris-HCl, pH 7.5,

1 mM PMSF and 100 mM benzamidine-HCl. AroAH₆ was concentrated to 1 mM and stored in 50 mM HEPES buffer, pH 7.5, on ice. Protein concentration was determined from A₂₈₀ by using ε_{280} = 3.55 * 10⁴ M⁻¹ cm⁻¹ (9, 55).

2.1.2 AroA Active Site Titration

The concentration of active AroAH₆ (as distinct from the protein concentration) was determined using a fluorescence titration with the substrate S3P. S3P binding in the presence of glyphosate causes a decrease in intrinsic Trp fluorescence (*13*, *34*, *56*). A mixture containing 0.5 μ M AroAH₆, 250 μ M glyphosate, 50 mM HEPES pH 7.0, 50 mM KCl, was prepared in a 2 mL reaction volume and allowed to equilibrate at room temperature. The excitation wavelength used was 280 nm, with emission at 360 nm. S3P concentration was increased by increments of 49.5 nM. The fluorescence of the mixture was allowed to stabilize each time before adding the next aliquot of S3P. Readings were taken until no more decrease in fluorescence was observed upon addition of S3P. Raw fluorescence reading were corrected for dilution during the experiment.

2.1.3 PPDK Expression and Purification

Isotopically labeled PEPs were synthesized from pyruvate and ATP using pyruvate phosphate dikinase (PPDK). The PPDK gene, cloned in pACY184, was a gift from Dr. Dunaway-Mariano (University of New Mexico). It was transformed

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in JM101 cells (*57*). Transformed cells were selected using LB agar plates with 10 μ g/mL tetracycline. Large scale cultures (6 × 1 L, LB + 10 μ g/mL tetracycline) were inoculated with 5 mL of overnight culture and grown at 37 °C for 16 hrs. Cells were collected by centrifugation at 10,000 × g for 10 min at 4 °C and resuspended in Buffer A (80 mM imidazole, 2.5 mM Na₂EDTA, 2 mM DTT, 75 mM KCI, pH 6.8). Cells were lysed with 3 cycles through the French press at 10,000 psi. Ammonium sulphate was added to the cell lysate to 50% saturation and the mixture was centrifuged at 25,000 × g for 10 min. The pellet was discarded and ammonium sulphate was added to 62% saturation and centrifuged at 25,000 × g for 10 min. The pellet was loaded on a 20 mL DEAE-Sephadex column for PPDK purification. PPDK was purified using a gradient of 100% Buffer A to 100% Buffer B (500 mM KCI, 20 mM imidazole, 2.5 mM EDTA, 1mM DTT, pH 6.4) over 40 min at 2 mL/min (*57*). PPDK was stored at -20 °C.

2.2 MG/AM Assay for Phosphate

The Malachite Green / ammonium molybdate (MG/AM) assay is a spectrophotometric assay used to determine inorganic phosphate concentrations (58). Briefly, 0.045% of Malachite Green solution was mixed with 4 M HCl containing 4.2% ammonium molybdate in a ratio of 1:3. The sample being assayed (100 μ L) was added to 800 μ L of MG/AM solution. After 1 minute, 100 μ L of 34% sodium citrate solution was added to the reaction to quench the

reaction and stabilize the colour development. To determine the concentration of inorganic phosphate, A_{660} was measured and compared to a phosphate standard curve.

2.3 Isotopologues Synthesis

2.3.1 [3,3-²H₂]PEP Synthesis

[3,3-²H₂]PEP was synthesized by allowing PEP to exchange protons with deuterium from D₂O, catalyzed by AroA (*40*). PEP (10 mM) was reacted with 0.1 mM S3P using 1 μ M AroA enzyme in the presence of 10 mM inorganic phosphate. The reaction mixture was exchanged with D₂O three times before addition of AroA to ensure that no H₂O molecules would be present. Synthesis of [3,3-²H₂]PEP was monitored by NMR, and the product was purified by anion exchange chromatography using a 20 mL Q-Sepharose column with a gradient of 100 mM to 600 mM NH₄⁺HCO₃⁻, pH 10, over 30 min. The buffer was lyophilized and the remaining PEP was dissolved in H₂O. The product was verified by mass spectrometry. The concentration of PEP was determined using the MG/AM assay by reacting it with AroA in the presence of excess S3P. The amount of phosphate formed corresponded to the PEP concentration (*41*, *59*).

2.3.2 [3-¹³C], [2-¹³C], [1-¹³C] PEP Synthesis

[3-¹³C], [2-¹³C], and [1-¹³C] pyruvates were purchased from Cambridge Isotopes Labs. Labeled PEP was synthesized by converting labeled pyruvate to PEP using PPDK, which catalyzes the following reaction in Scheme 1

Pyruvate + ATP + P_i
$$\xrightarrow{\text{PPDK}}$$
 PEP + AMP + PP_i $\xrightarrow{\text{PPiase}}$ 2 P_i
 H_2O Scheme 1.

Briefly, 3.7 mM of $[3-^{13}C]$ pyruvate, 3.7 mM ATP, 50 mM Pi, 10 mM MgCl₂, 5 mM KCl were used to synthesize PEP using 10 U of inorganic pyrophosphatase (PPiase) and 0.3 U of PPDK (9). $[3-^{13}C]$ PEP was purified under the same conditions as $[3,3-^{2}H_{2}]$ PEP. Synthesis of $[3-^{13}C]$ PEP was verified by mass spectrometry.

2.3.3 S3P Synthesis and Purification

S3P was synthesized by reacting 100 mM shikimic acid, 10 mM ATP, 100 mM PEP, 100 mM MgCl₂, 100 mM sodium tungstate, 1 M HEPES, 1 M KCl, and crude extract of shikimate kinase (AroK). The pH was adjusted with 10 M KOH to pH = 8.0 before adding the enzyme. The reaction was stopped by heating for 2 min at 95 $^{\circ}$ C and centrifuging. The supernatant was removed and diluted 10-fold before purification. S3P was purified by anion exchange chromatography using a Q-Sepharose column. The buffer gradient used was 50 mM to 1 M ammonium formate, pH 8.0, over 30 min at a flow rate of 0.5 mL/min (9). S3P eluted at 16

min. The buffer was lyophilized and the concentration was determined using the MG/AM assay by reacting it with AroA in the presence of excess PEP. The amount of phosphate formed corresponded to the S3P concentration.

2.4 KIE Measurements by Mass spectrometry

2.4.1 Mass Spectrometer and Conditions

KIEs were determined using a tandem quadrupole instrument (Quattro Ultima, Micromass). Continuous flow infusion was used to introduce samples for ionization by microelectrospray. Data were collected in negative ion mode for 20 min. Capillary voltage was set to -3.00 kV and cone voltage was set to 35 V. Cone gas flow rate was optimized for each run and desolvation gas was not used. Resolution parameter was set to 15 on the software used to determine KIEs. This ensured baseline resolution between peaks, with a width at half-height (w_h) of m/z = 0.7.

A standard curve of PEP isotope ratios was created using PEP and $[3,3-^{2}H_{2}]PEP$. Solutions containing varying fractional ratios of PEP and $[3,3-^{2}H_{2}]PEP$ were prepared and used to optimize conditions to measure KIEs. Fractional ratios of PEP calculated by equation (5),

$$f(\text{PEP}) = \frac{[\text{PEP}]}{[\text{PEP}] + [[3,3^{-2}\text{H}_2]\text{PEP}]]}$$
(5),

ranged from 0.5 to 0.9. Conditions were optimized to give a high signal to noise ratio and a slope of 1 for the standard curve. Peak areas from the spectra collected were used to calculated the observed ratios.

2.4.2 Solvents

PEP was dissolved in two different solvents to determine which solvent gave the best signal to noise ratio. The concentration of PEP used was 33 μ M and was kept constant for both solvents. Solvents tested were water, and 50% aqueous methanol with 0.1% formic acid.

2.4.3 Flow rate

Solutions of PEP were dissolved in 50% aqueous methanol with 0.1% formic acid and injected into the mass spectrometer at different flow rates. Flow rates tested were 1 μ L/min and 2 μ L/min. The flow rate that gave the best correlation between theoretical and observed ratios was chosen to determine the KIEs. The concentration of PEP used while optimizing flow rates was 3.3 μ M.

2.4.4 Concentration

The concentration of PEP was varied in order to get the best correlation between theoretical and observed ratios with the least concentrated solution of PEP. Concentrations of PEP were 0.33 μ M, 1.65 μ M, 3.3 μ M and 33 μ M. PEP

was dissolved in 50% aqueous methanol with 0.1% formic acid. Concentrations were tested at a flow rate of 2 μ L/ min.

2.4.5 Standard Curve with Smaller Changes in Ratios

A standard curve of PEP isotope ratios was created using PEP and $[3,3-{}^{2}H_{2}]$ PEP. Solutions containing varying fractional ratios of PEP and $[3,3-{}^{2}H_{2}]$ PEP were prepared and used to optimize conditions to measure KIEs. Fractional ratios of PEP calculated by equation (5), ranged from 0.5 to 0.9. Conditions were optimized to give a high signal to noise ratio and a slope of 1 and low standard of error for the standard curve between the theoretical and observed fractional isotope ratios of PEP.

Conditions that gave the best correlation between theoretical and observed isotope ratios using the least amount of PEP were tested with a smaller range of fractional ratios. Fractional ratios ranged from 0.50 to 0.52. Standard solutions were made by making a mixture of 50 μ M each of unlabeled and [3,3-²H₂]PEP. Five 20 μ L aliquots were taken and 10 to 50 μ L of 1.0 μ M PEP was added to each. Mixtures were lyophilized and reconstituted in 100 μ L of 50% aqueous methanol + 0.1% formic acid. The standard solutions were injected into the mass spectrometer at 2 μ L/min and data was collected over 10 min. Mass spectrometer tubing was thoroughly cleaned with 50% aqueous methanol between each run.

2.5 Enzymatic KIEs

2.5.1 Computer Simulation of Reaction conditions

Because the AroA reaction is fully reversible, it was necessary to ensure that, under the conditions used to measure KIEs, there was no significant extent of reverse reaction (which would change the isotope ratios). KinTeKSim, a software program (*60*), was used to simulate an AroA reaction to determine how much PEP would be formed from the backward reaction. Microscopic rate constants for the AroA reaction as determined by Anderson and Johnson were preprogrammed (*14*).

The kinetic mechanism for AroA is described in equation (6):

$$E + PEP \implies E \cdot PEP \implies E \cdot PEP \cdot S3P \implies E \cdot THI$$

$$\downarrow \downarrow$$

 $EPSP + Pi + E \implies E \cdot EPSP + Pi \implies E \cdot EPSP \cdot Pi$

(6)

To measure the backwards reaction, P_i was "tagged" by immediately converting it to P_i^* . P_i^* was identical to P_i . Any P_i^* that was reacted with EPSP to re-form PEP^{*} was made unreactive to prevent reaction again with AroA. At the end of the simulation PEP^{*} was quantitated and compared to residual PEP.

Initial concentrations of each were entered as follows: [E] = 0.05 μ M, [PEP]= 0.4 mM, [S3P] = 2 mM.

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2.5.2 Time to Reach 50% Completion

A 150 μ L reaction mixture containing 50 mM citric acid, pH 5.0, 0.4 mM PEP was reacted with 2 mM S3P using 0.05 μ M AroA. The extent of reaction was monitored by the MG/AM assay. Each 10 μ L aliquot was diluted 10-fold and added to 800 μ L of MG/AM solution at 1 min intervals for 30 min. [P_i] was calculated using the standard curve for the MG/AM assay and the extent of the reaction was determined.

2.5.3 Extent of Reaction by HPLC

When measuring KIEs, it is necessary to know the extent of reaction with high precision, better than attainable using the MG/AM Assay. This was done using an HPLC assay. Aliquots (10 μ L) of the reaction mixture were diluted 20-fold and analytical injections of 200 μ L were made on the HPLC (626 pump, λ_{240} , Waters). The reaction mixture was separated by anion exchange chromatography using a 1 mL Mono-Q column with a gradient of 100 mM to 600 mM NH₄⁺HCO₃⁻, pH 10, over 30 min. The extent of the reaction was reported as the fractional extent of reaction (equation 7):

$$f = \frac{[\mathsf{EPSP}]}{[\mathsf{EPSP}] + [\mathsf{PEP}]} \tag{7}$$

The ε_{240} of EPSP, PEP and S3P differ from each other. Peak areas were corrected for the difference in absorptivities before calculating the extent of

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reaction. Absorptivity ratios were determined between PEP and S3P by injecting equal amounts of PEP and S3P. The absorptivity ratio between EPSP and S3P was determined by reacting known amount of PEP with known amount of excess S3P using AroA. Since S3P and PEP react in a 1:1 ratio the amount EPSP synthesized was calculated and an absorptivity ratio between S3P and EPSP was determined. To determine the ratio between PEP and EPSP, a known amount of S3P was reacted with a known amount of excess PEP using AroA.

2.5.4 Enzymatic KIEs Measurement

KIEs were measured by mass spectrometry. A 500 μ L reaction containing 400 μ M PEP, 2 mM S3P in 50 mM acetate buffer, pH 5.0, was split 2:1 into two Eppendorf tubes. AroAH₆ (0.05 μ M) was added to the reaction containing 2/3 of the reaction mixture while the other had no enzyme. The reaction in the tube containing the enzyme was taken to approximately 50% completion. The reaction was quenched by adding 20 μ L of 2 N KOH and 50 μ L of KOH-saturated isopropanol. The denatured enzyme was extracted using 250 μ L of chloroform. PEP in the aqueous layer was purified using anion exchange chromatography using the Mono-Q column with a gradient of 100 - 600 mM of ammonium bicarbonate, pH 10, which was later lyophilized. The remaining PEP was dissolved in 50:50 aqueous methanol with 0.1% formic acid to a final concentration of 100 μ M before injecting on the mass spectrometer at a flow rate

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identical (Fig. 11).



of 2 µL/min for 20 min. Treatment of the control containing no enzyme was

Figure 11: Flow chart of KIE determination.

KIEs were calculated using equation (8):

$$\text{KIE} = \frac{\ln \left[\frac{(1-f)(1+r_0^{-1})}{(1+r_i^{-1})} \right]}{\ln \left[\frac{(1-f)(1+r_0)}{(1+r_i)} \right]}$$
(8),

where f is the fractional extent of reaction, r_o and r_i are the ratios of unlabeled:

labeled PEP before and after the reaction, respectively (52).

2.6 Acid-catalyzed PEP Breakdown

2.6.1 Rates of Acid-catalyzed PEP hydrolysis

Rates for PEP breakdown under acidic conditions were initially determined using 2 M HClO₄ at 35 $^{\circ}$ C. PEP (300 nmol, 80 µg) was added to 600 µL of 2 M HClO₄. Aliquots (50 µL) were taken at different time points and added to 10 µL of 10 M KOH to quench the reaction, then 40 µL of H₂O was added to the quenched reaction to bring the volume to 100 µL. The reaction was mixed well and then centrifuged at 14, 000 × g for 1 min to pellet the precipitated KClO₄. Part of the supernatant (75 µL) was then diluted 100-fold to 7.5 mL, 100 µL of which was used to determine the phosphate concentration by MG/AM assay. Rates were similarly determined using 8.3 M HClO₄ at 35 $^{\circ}$ C and 75 $^{\circ}$ C, and using 5.6 M HClO₄ at 35 $^{\circ}$ C. Rate constants for PEP breakdown in methanol were determined under the same conditions as PEP hydrolysis in H₂O.

2.6.2 Phosphate Assay

The MG/AM assay was used to follow P_i formed from PEP breakdown. The non-enzymatic reactions were run in perchloric acid and neutralized with KOH, which leads to a large amount of insoluble KCIO₄ salt. Samples for the phosphate standard curve were prepared in the same way to ensure that any effects of KCIO₄ were adequately controlled for. Known amounts of phosphate were added to 50 µL of perchloric acid of a known concentration, then 10 µL of

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10 M KOH was added to neutralize the acid and 40 μ L of H₂O was added so that the volume was 100 μ L. The white precipitate formed was removed by centrifugation for 1 min at 14,000 × g. Part of the supernatant (75 μ L) was diluted to 7.5 mL, of which 100 μ L was used for the MG/AM assay. To the 100 μ L of diluted supernatant, 800 μ L of MG/AM solution was added, then 100 μ L of 34% sodium citrate to neutralize the solution. A₆₆₀ was read after 30 min.

2.6.3 Does PEP Exchange Protons with Solvent?

Solid PEP (1 μ mol, 0.267 mg) was added to 50% perchloric acid and 50% D₂O. The mixture was kept at 35 °C for 12 hours. The remaining PEP was purified by anion exchange chromatography on the Mono-Q column under the same conditions as PEP purification for KIE reactions. The buffer was removed by lyophilization and the purified PEP was reconstituted in 1 mL of 50% methanol, 10 μ L of which was injected on the electrospray mass spectrometer to detect the formation of singly or doubly deuterated PEP.

2.6.4 C-O versus P-O Bond Cleavage in PEP Breakdown

PEP is known to breakdown predominantly through P-O cleavage under moderately acidic conditions (pH>0). C-O *versus* P-O bond cleavage was determined by performing the reaction in methanol. It is known that P-O bond cleavage in methanol yields monomethyl phosphate (*12*). PEP (300 nmol, 80 μ g) was weighed into an Eppendorf tube containing 8.3 M HClO₄, and 50%

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methanol. The reaction mixture was incubated at 35 $^{\circ}$ C for approximately 7 hours. Any monomethyl phosphate formed during this reaction was purified from pyruvate and phosphate. The gradient used to purify monomethyl phosphate from phosphate was 20 mM – 500 mM KCl in 10 mM KOH, pH 12, for 30 min at a flow rate of 0.5 mL/min using a Mono-Q column. The amount of monomethyl phosphate was determined by treating eluate fractions with calf alkaline phosphatase as stated in section 2.6.5. This experiment was repeated with 5.8 M HClO₄ containing 50% methanol.

2.6.5 Limit of Detection and Purification of Monomethyl Phosphate

A 100 μ L solution containing 1 mM phosphate and 10 μ M monomethyl phosphate was injected on the HPLC. The goal was to determine the least amount of monomethyl phosphate that could be detected when purified from phosphate such that the monomethyl phosphate was 1% of the total phosphate. Fractions of 250 μ L were collected for the first 20 min, and 100 μ L of eluate from each aliquot was used to detect inorganic phosphate by the MG/AM assay. The remaining eluate, 100 μ L, was incubated with 3U of calf alkaline phosphatase for 5 hours and used to detect phosphate from the breakdown of monomethyl phosphate by the MG/AM assay.

2.6.6 Does Monomethyl Phosphate Break Down under Acidic Conditions at 35°C?

Monomethyl phosphate was added to 100 μ L of 5.8 M HClO₄ and 50% methanol and kept at 35 ^oC for approximately 10 hours. The reaction mixture was split into two and the amount of phosphate was detected before and after the reaction. Aqueous 10 M KOH was added until the pH of the reaction mixture was above pH 7, and 40 μ L of H₂O was then added to the reaction mixture. The mixture was centrifuged, and 75 μ L of the supernatant was diluted to 7.5 mL, 100 μ L of which was used for the MG/AM assay to detect formation of phosphate.

2.6.7 Extent of PEP Reaction

Aliquots (10 μ L) of the non-enzymatic KIE reaction mixture were diluted 20-fold and analytical injections of 200 μ L were made on the HPLC. The fractional extent of the reaction (*f*) was reported as in equation (9):

$$f = \frac{[\text{pyruvate}]}{[\text{pyruvate}] + [\text{PEP}]}$$
(9)

The ε_{240} of PEP and pyruvate are different; peak areas were corrected for the difference in absorptivities. Absorptivity ratios between PEP and pyruvate were determined by injecting mixtures containing different ratios of PEP and pyruvate.

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2.6.8 Acid-Catalyzed KIE Measurement

KIEs were measured by mass spectrometry using a method similar to the enzymatic KIEs (Fig. 11). A 500 μ L reaction mixture containing 200 μ M of each isotopologue of PEP and 8.3M HCIO₄ was prepared. For the 'no reaction' samples, 250 μ L of the reaction mixture was quenched immediately after the addition of PEP to the acid by adding 10M KOH until the pH was 12. The remaining 250 μ L of the reaction mixture was incubated for 6 hours at 35 °C and quenched in the same manner as the 'no reaction' samples. Both samples were diluted 10-fold. Samples were purified, lyophilized and reconstituted in 50% methanol. Initial ratios of labeled:unlabeled PEP were compared with ratios after 50% reaction completion to determine the KIEs.

3 RESULTS

3.1 AroA Purification

AroAH₆ was expressed, purified, and characterized previously by our lab (9). AroAH₆ used in this study was produced by the same method. His-tagged AroA, AroAH₆ was purified by metal affinity chromatography using a chelating column charged with Ni²⁺. Its purity was tested by 10% SDS-PAGE and visualizing by Commassie Blue staining. AroAH₆ was 99% pure. The molecular weight of AroAH₆ is 48546 Da. A 6 L culture gave approximately 60 mg of protein.

3.2 Active Site Titration of AroA

The concentration of active enzyme, as distinct from the protein concentration, was determined by a fluorescence titration (*56*). In the presence of a large excess of glyphosate, S3P binds quantitatively with AroAH₆, causing a decrease in the intrinsic Trp fluorescence. From the fluorescence titration, a protein concentration of 0.5 μ M AroAH₆ bound 0.34 μ M S3P, indicating that it was 68% active (Fig. 12).



Figure 12: AroAH $_6$ active site titration showing the decrease in fluorescence at 360nm as the concentration of S3P increases.

3.3 $[3,3-^{2}H_{2}]$ PEP Synthesis

A reaction mixture containing PEP, S3P and P_i was added to D_2O in the presence of AroA as shown in Scheme 2. The catalytic amount of S3P used ensured that equilibrium lay to the left and that 99 % of the PEP would eventually be converted to $[3,3-^2H_2]PEP$.



Scheme 2,

Synthesis of $[3,3-{}^{2}H_{2}]$ PEP in D₂O was monitored by proton NMR (Fig. 13). The bottom spectrum shows the reaction mixture before AroA was added. The peaks at around 1.8 ppm correspond to protons of cyclohexylamine which is used as a counterion in commercial PEP. The peak at 3.0 ppm corresponds to a contaminant in the reaction mixture. The peak at 4.8 ppm is the solvent peak. The peaks at 5.1 and 5.3 ppm correspond to protons of PEP which were exchanged with solvent deuterium to make $[3,3-{}^{2}H_{2}]$ PEP.



Figure 13: NMR spectra of $[3,3-{}^{2}H_{2}]$ PEP synthesis reaction in D₂O. (bottom) Reaction solution before the enzyme was added, and (top), reaction 48 hrs after the enzyme was added.

The top spectrum shows the reaction contents 48 hrs after AroAH₆ was

added. Peaks corresponding to PEP protons are smaller, indicating the protons

have been exchanged with deuterons. Solvent contaminant and the

cyclohexylamine peaks are however still present.

Peak c of the cyclohexylamine was used as an internal standard to calculate the extent of the reaction. Ratios of the internal standard peak height to peak height corresponding to PEP, before and after the addition of the enzyme, were used to determine the extent of exchange of PEP protons with solvent deuterium. PEP was purified by HPLC and was injected into the mass spectrometer to verify complete synthesis of [3,3- ${}^{2}H_{2}$] PEP.

At equilibrium, [PEP] remained 9.9 mM. Each time a molecule of PEP was converted to EPSP, and vice versa, it exchanged a hydron with solution. Thus, with extended incubation times, the PEP protons became equilibrated with solvent deuterons. When all protons were exchanged with solvent deuterons, peaks due to PEP protons could no longer be seen in the NMR spectrum.

3.4 Factors Affecting the Correlation between Theoretical Relative Ratios and Observed Relative Ratios Using Mass Spectrometry

Using mass spectrometry for quantitative purposes is not very popular and can be quite difficult. It was therefore important to first determine if mass spectrometry could be used to determine kinetic isotope effects. A number of variables were manipulated in order to optimize the mass spectrometer conditions. Conditions tested were flow rate, concentration, solvent and the method used to calculate isotope ratios. After each parameter was adjusted, the observed isotope ratios were compared to theoretical values. Standard curves of isotope ratios were created by measuring isotope ratios,

(r = (PEP/ (PEP + $[3,3^{-2}H_2]PEP$)) for solutions containing known ratios of PEP and $[3,3^{-2}H_2]PEP$. The goal was to get a slope of the observed line to equal 1, with low standard error.

3.4.1 Flow Rate

Results show that correlation between theoretical and observed isotope ratios is influenced by the flow rate (Fig. 14). The standard error calculated for the higher flow rate, 2 μ L/ min, is 0.2 whereas that for 1 μ L/min was 0.4. This indicated that ratios obtained at higher flow rates are more precise (Table 1). Also, the signal for each of the samples run at 2 μ L/min was much more stable than for samples run at 1 μ L/min. Samples were run using [PEP] = 1.65 μ M. The concentration had not been optimized prior to this experiment.



Figure 14: Influence of flow rate on the measurement of isotope ratios between labeled and unlabeled PEP. An ideal relationship is indicated by the dashed line.

Flow Rate	Slope	Std. Error	
1 μL/min	0.9	0.4	
2 μL/min	0.8	0.2	

Table 1: Slope and Standard Error for Different Flow Rates

3.4.2 Concentration

Concentration also had an effect on the standard error and the correlation between theoretical and observed isotope ratios. As concentration of PEP increased, the slope of the "observed ratios" line approached 1 and the standard error improved significantly (Fig. 15). Using a PEP solution of 0.33 μ M concentration gave a slope of 0.4 and an error value of 0.3 (Table 2). Although using 3.3 μ M PEP solution gave results suitable for KIE measurement, a higher concentration of PEP was used for determining KIEs. Using a higher concentration of PEP does not affect the correlation between theoretical and observed isotope ratios or the standard of error greatly (Fig. 16). However, using a higher concentration allows more leeway for error in the concentration of PEP solution used for determining KIEs.



Figure 15: Influence of concentration on the measurement of isotope ratios between labeled and unlabeled PEP.

Table 2: Slope and Standard Error for Different Concentrat
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[PEP] (µM)	Slope	Std. Error
0.33	0.4	0.3
1.65	0.8	0.2
3.3	0.95	0.04
33	1.01	0.03



Figure 16: Effect of concentration on precision of isotope ratio measurement. The dotted line is included only as a visual aid.

3.4.3 Solvent

The solvent had a significant effect on signal, which improved correlation between theoretical and observed ratios. Dissolving PEP in 50% methanol with 0.1% formic acid increased the signal by 9-fold compared to water alone. [PEP] was 33 μ M. The error value and the slope of the lines reflect this. The standard error was 0.03, with a slope of 1.01 when PEP was dissolved in 50% methanol with 0.1% formic acid whereas, the standard error was 0.3 with a slope of 0.5 when PEP was dissolved in water alone (Fig. 17, Table 3).

consistently the same under real assay conditions, occasionally giving inaccurate measurements of peak height.

Conditions that gave optimal results were using > 33 μ M PEP dissolved in 50% methanol and 0.1% formic acid, at a flow rate of 2 μ L/min.



Figure 18: Comparison on the type of measurement for determining isotope ratios between labeled and unlabeled PEP accurately.

3.4.5 Correlation with a Smaller Range of Ratios

Ratios of labeled and unlabeled PEP having a range from 0.494 to 0.513 were measured. This was done to ensure that conditions used to determine isotope ratios over a large range could also be used to measure small changes in isotope ratios expected while measuring KIEs. The conditions gave a good correlation between observed and theoretical values indicating that the conditions determined could accurately measure KIEs. The slope was 0.97 ± 0.08 (Fig. 19).



Figure 19: Measurement of isotope ratios between labeled and unlabeled PEP using a narrow range of isotope ratios. The discrepancy between theoretical and observed ratios represents normal pipetting error in preparing PEP mixtures. The important feature of this plot is the linearity of change in isotope ratios, with slope near 1.

3.5 Determining the Reaction Conditions to Measure KIEs

The AroA reaction is freely reversible, with $K_{eq} = 180 (56)$. To determine KIEs on the AroA reaction by measuring residual amount of labeled and unlabeled PEP, it was imperative to make sure that the PEP measured was solely due to the initial PEP added and that none of the PEP present was from the backward reaction. Contamination with PEP from the backward reaction would alter the observed KIE.

A numerical simulation was run to determine the concentration of PEP, S3P and AroA that would meet the criteria. It is important to note that it is the actual molecule of PEP that is in question and not the concentration of PEP. In order to follow individual PEP molecules, Pi formed from the forward reaction was "tagged" to allow it to be followed in the reverse reaction. The concentrations used in the simulation experiment were 400 μ M PEP, 2mM S3P and 0.5 μ M AroA. The amount of PEP from the backward reaction was determined to be 0.2% of the initial concentration of PEP when 99% of the PEP was reacted. This suggests that the amount of PEP present from the backward reaction would be even lower when the reaction is at 50% completion.

3.6 Determined the Time for 50% Reaction Completion

It was necessary to find, experimentally, reaction conditions that would give 50% reaction at pH 5.0. The extent of the reaction was monitored by using the MG/AM assay, which measures the amount of inorganic phosphate in solution. The reaction was monitored to determine the time at which the reaction would reach 50% completion, and also to verify that, at equilibrium, the concentration of PEP was as low as determined by the simulation experiment. The time at which the reaction reached 50% completion was 1.5 min. The equilibrium concentration of inorganic phosphate was approximately 400 μ M, showing that concentration of PEP is low at equilibrium (Fig. 20).



Figure 20: Time course of the AroA reaction with 5 nm AroA, 400 µM PEP and 2 mM S3P.

3.7 Determining the Absorptivity ratio for S3P:PEP:EPSP

The ε_{240} of EPSP, PEP and S3P are different from each other. The ratios of absorptivities were determined by injecting known amounts of PEP, S3P and EPSP onto the Mono-Q anion exchange column, using the same gradient used for purification of PEP for determining KIEs. The ε_{240} ratio for PEP:S3P was 1.24:1, and ε_{240} ratio for PEP:EPSP was 1:2.18, and for S3P:EPSP it was 1:2.58. Therefore, the ε_{240} ratio for S3P:PEP:EPSP was 1:1.24:2.58. These absorptivities were used when determining the extent of reaction by HPLC when determining KIEs.

3.8 [3,3–²H₂] PEP KIE Measurement for AroA-catalyzed Reaction

The $[3,3-^{2}H_{2}]$ PEP KIEs were measured by collecting spectra over an m/z range of 165 and 170 which gave much better standard errors compared to KIEs

measured over an m/z range of 160 and 170. The $[3,3-{}^{2}H_{2}]PEP$ KIE was determined to be 0.985 ± 0.003, an inverse isotope effect. KIEs measured were within the expected range of 0.70-1.5 for deuterium secondary isotope effects (61) (Table 4).

Sample #	Extent of Reaction (<i>f</i>)	Ratio of unreacted PEP (r _o)	Ratio of reacted PEP (<i>r</i> _i)	KIE
1	0.86	1.176	1.206	0.987
2	0.86	1.156	1.183	0.988
3	0.46	0.827	0.836	0.983
4	0.36	0.847	0.855	0.980
5	0.36	0.913	0.920	0.984
			Average	0.984 ± 0.003

Table 4: Results for [3,3-²H₂]PEP KIEs for AroA-catalyzed Reaction

3.9 KIE measurement on [3-¹³C]PEP for AroA-catalyzed Reaction

The [3- ¹³C]PEP KIE was determined to be 0.97 \pm 0.01. [3- ¹³C]PEP also had an inverse isotope effect. The expected range for primary [3- ¹³C]PEP KIEs is from 0.95 to 1.05 (*61*). KIEs measured on [3- ¹³C] PEP falls within this range (Table 5).

Sample #	Extent of Reaction (<i>f</i>)	Ratio of unreacted PEP (<i>r₀)</i>	Ratio of reacted PEP (<i>r_i</i>)	KIE
1	0.87	1.027	1.094	0.969
2	0.79	0.884	0.938	0.963
			Average	0.97± 0.01

Table 5: Results for [3- ¹³C]PEP KIEs

3.10 Rates for PEP Breakdown in Acid

Reaction rates were initially determined using 2 M HClO₄ at room temperature. The reaction was very slow and therefore the acid concentration was increased to 8.3 M HClO₄. The reaction was also conducted at 35 ^oC and 75 ^oC. The rate constant for PEP hydrolysis at 35 ^oC was determined to be (1.6 ± 0.1) * 10⁻³ min⁻¹ and the reaction rate constant for PEP hydrolysis at 75 ^oC was determined to be (9 ± 2) * 10⁻² min⁻¹(Fig. 21).



Figure 21: Rate constants determined for PEP hydrolysis in 8.3 M HClO₄ at 35 $^{\circ}$ C and 75 $^{\circ}$ C
3.11 Do PEP Protons Exchange with Solvent Under Acidic Conditions?

During the acid-catalyzed hydrolysis of PEP, it is possible, that an equilibrium is established between PEP and the oxocarbenium ion intermediate. If this is true, it is possible for PEP to exchange its protons with the solvent. Since KIEs are determined by measuring a ratio between unlabeled and labeled PEP, exchange of PEP protons with the solvent would alter the [3,3-²H₂]PEP KIEs. To determine if PEP protons do indeed exchange with solvent protons, formation of singly or doubly labeled PEP was detected by mass spectrometry after 50 % acid-catalyzed hydrolysis of PEP in D₂O.

PEP does not appear to exchange protons with solvent. Remaining PEP, after incubation in 50% D_2O and 50% $HCIO_4$ at 35 ^{0}C for 12 hours, was purified and was injected onto the mass spectrometer to detect formation of either singly or doubly deuterated PEP. The mass spectra do not show an increase in the peaks at m/z = 168 or 169 which would correspond to deuterated PEP (Fig. 22).



Figure 22: Mass spectra of purified PEP before and after incubation in 8.3 M HClO₄ in 50% D_2O . Mass spectrum of PEP after incubation in 8.3 M HClO₄ does not show an increase in the peaks at m/z =168 or 169.

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3.12 PEP Hydrolysis Rates in H₂O and Methanol

PEP breakdown can proceed by either C-O bond cleavage or P-O bond cleavage to form pyruvate and phosphate. If acid-catalyzed PEP breakdown takes place by P-O bond cleavage, then reaction in the presence of methanol will cause it to react with the metaphosphate intermediate to produce monomethyl phosphate (*62*). However, inorganic phosphate produced by C-O bond cleavage is unreactive to methanol and there is no formation of monomethyl phosphate. In order to ensure that formation or lack of formation of monomethyl phosphate reflects the mode of PEP breakdown and not the difference in rates of PEP breakdown in H₂O and 50% methanol, rates were determined in both solvents. The rate of PEP breakdown was determined by measuring the rate of phosphate formation with the MG/AM assay. The rates of PEP hydrolysis in H₂O and 50% methanol were approximately the same. The rate constant in water was $k = 0.7 \pm (0.3) \times 10^{-3} \text{ min}^{-1}$, and the rate constant for PEP hydrolysis in 50 % methanol was $k = 0.7 \pm (0.4) \times 10^{-3} \text{ min}^{-1}$.

3.13 Assay for Detecting Monomethyl Phosphate

The MG/AM assay used to detect inorganic phosphate cannot detect monomethyl phosphate. Alkaline phosphatase cleaves monomethyl phosphate to produce inorganic phosphate which can then be detected by the MG/AM assay. The accuracy and reliability of this method was tested by determining the correlation factor between observed phosphate formed by monomethyl

phosphate digestion with alkaline phosphatase and the expected yield of phosphate after monomethyl phosphate digestion with alkaline phosphatase. The correlation factor was 0.81, which is close to unity, indicating that this method was sensitive enough to detect monomethyl phosphate that may be produced during the acid-catalyzed PEP breakdown in 50% aqueous methanol (Fig. 23).



Figure 23: Correlation between the theoretical and observed values for monomethyl phosphate measured after digestion of monomethyl phosphate by alkaline phosphatase. Inorganic phosphate was measured by MG/AM assay after alkaline phosphatase treatment.

3.14 **Purification of Monomethyl Phosphate and Phosphate**

Purification of monomethyl phosphate and phosphate was accomplished by using a gradient of 20 mM to 500 mM KCl in 10 M KOH, pH 12, on a Mono-Q anion exchange column. Monomethyl phosphate eluted at a retention time of 8 min, and phosphate eluted at a retention time of 11 min (Fig. 24). The smallest amount of monomethyl phosphate that could be detected and successfully purified was 10 nmoles from 1 μ mol of phosphate.



Figure 24: Chromatogram of purification of inorganic phosphate from monomethyl phosphate. Retention time for monomethyl phosphate is around 8 min and for phosphate is around 11 min.

3.15 Determining the Cleavage Site of PEP under Acidic Conditions using 5.8 M HCIO₄

To determine if the acid-catalyzed PEP hydrolysis proceeded through C-O bond or a P-O bond cleavage, PEP was hydrolyzed under acidic conditions containing 50% water and 50% methanol. After purification of the reaction mixture by HPLC, eluate fractions were treated with alkaline phosphatase. Approximately 10% of the product was determined to be monomethyl phosphate (Fig. 25). This indicates that the PEP hydrolysis undergoes a C-O bond cleavage 80% of the time, and that 20% of the time it undergoes a P-O bond cleavage when hydrolyzed using 5.8 M HClO₄. However, monomethyl phosphate was not formed when [HClO₄] was increased to 8.3 M HClO₄ indicating that decreasing the pH promotes C-O bond cleavage rather than a P-O bond cleavage for PEP hydrolysis (Fig. 26).



Figure 25: Formation of monomethyl phosphate using 5.8 M HClO₄ and methanol, indicating P-O bond cleavage. The peak at approximately 9 min was from monomethyl phosphate while phosphate eluted at approximately 12 min.



C-O or P-O Cleavage for PEP Hydrolysis in 8.3 M HCIO4 and Methanol

Figure 26: PEP hydrolysis using 8.3 M HClO₄ and methanol shows no formation of monomethyl phosphate, indicating C-O cleavage for PEP hydrolysis.

3.16 Formation of Monomethyl Phosphate under Acidic Conditions

In order to determine the cleavage site for PEP hydrolysis, it was first important to determine whether or not monomethyl phosphate was formed under acidic conditions from inorganic phosphate. Inorganic phosphate was added to 8.3 M perchloric acid and incubated at 35 ^oC for 7 hrs. The reaction mixture was purified on the HPLC and aliquots were treated with alkaline phosphatase to determine any formation of monomethyl phosphate. A peak corresponding to the formation of monomethyl phosphate was not observed (Fig. 27).



Formation of Monomethyl Phosphate in 8.3M HCIO4

Figure 27: No formation of monomethyl phosphate under conditions used for determining C-O or P-O bond cleavage for PEP.

3.17 Breakdown of Monomethyl Phosphate under Acidic Conditions

In addition to formation of monomethyl phosphate, hydrolysis of monomethyl phosphate under acidic conditions was also tested. A monomethyl phosphate peak was not observed when PEP was incubated with 8.3 M HCIO₄ and 50% methanol. To ensure that the observed result was due to PEP breakdown via a

C-O bond cleavage and not due to monomethyl phosphate breakdown,

monomethyl phosphate was added to 8.3 M $HCIO_4$. It was incubated at 35 °C for 7 hours and the reaction mixture was purified on the HPLC. Inorganic phosphate produced by the breakdown of monomethyl phosphate was not detected by the MG/AM Assay when eluate fractions were treated with alkaline phosphatase.

3.18 [3,3⁻²H₂] PEP KIE Measurement for Non-Enzymatic Breakdown of PEP

The $[3,3-{}^{2}H_{2}]PEP$ KIE was determined to be 1.180 ± 0.009, a normal isotope effect. KIEs for secondary deuterated isotopes are generally within the range of 0.75 to 1.50 (*61*). The calculated value for $[3,3-{}^{2}H_{2}]PEP$ is within this range (Table 6).

Table 6: Results for $[3,3 - {}^{2}H_{2}]$ PEP KIE Measurement for non-enzymatic breakdown of PEP

Sample #	Extent of Reaction (<i>f</i>)	Ratio of unreacted PEP (<i>r</i> _o)	Ratio of reacted PEP (<i>r_i</i>)	KIE
1	0.53	0.459	0.519	1.184
2	0.53	1.198	1.350	1.170
3	0.53	1.239	1.412	1.187
			Average	1.180 ± 0.009

4 DISCUSSION

4.1 Factors Affecting the Correlation Between Theoretical Relative Ratios and Observed Relative Ratios using Mass Spectrometry

Parameters that were optimized in order to determine KIEs by mass spectrometry were flow rate, PEP concentration, solvent, and the method of calculating isotope ratios. Conditions used to determine KIEs by mass spectrometry were using a 100 μ M solution of pure PEP dissolved in 50% aqueous methanol, 0.1% formic acid, at a flow rate of 2 μ L/min. Ratios were calculated after integrating peaks corresponding to PEP and its isotopologue. In the future, these conditions can be used for determining KIEs for reactions where labeled molecule is negatively charged and is within a m/z range of 60 to 400. The resolution of peaks outside of this m/z range is poor under the conditions mentioned. It is however important that the stability of the signal and the signal to noise ratio remain the same as PEP.

4.2 KIE Measurements

The KIEs determined on the addition step of the enzymatic reaction cannot be interpreted with full certainty. Competitive KIEs reflect the difference in rate constants for two isotopologues on steps up to and including the first irreversible step. However, recent results show that the tetrahedral intermediate does not partition completely forward as determined previously. Thus, reacting PEP and

S3P with AroA at pH 5.0 does not ensure that the first irreversible step is the addition step. Hence, KIEs measured under those conditions reflect on both the addition and the elimination steps of the reaction. The KIEs reported here were measured prior to the new results showing that partitioning was not completely forward.

However, exogenously added THI does partition in the forward direction in a ratio of 4:1 at pH 5 (Fuzhong Zhang, personal communication). Therefore, KIEs measured on the enzymatic reaction reflect information predominantly on the addition step of the reaction. The experimental KIEs can be interpreted qualitatively to propose what the TS for the addition step may look like. An inverse isotope measured on $[3,3-^{2}H_{2}]$ PEP indicates that the C3 of PEP is protonated. The incoming hydrogen hinders the out-of-plane motion of the labeled hydrons, making the vibrational environment tighter at the transition state than in the reactant state. The large inverse isotope effect on $[3-^{13}C]$ PEP also indicated that C3 was protonated. Substitution of a stronger C-H σ -bond for a weaker π -bond from the C3=C2 double bond contributed to the inverse KIE, as did the change in geometry form sp² to sp³, which restricted bond bending motions.

4.3 Acid-catalyzed Breakdown of PEP

Examining the non-enzymatic breakdown of PEP, the intrinsic reactivity of PEP is revealed. By examining the transition state of the non-enzymatic and

enzymatic breakdown of PEP, we can study and understand how the enzyme modifies the transition state structure or lowers the activation energy in some other way. It is important to note that the transition states for the two reactions are not necessarily the same.

During the AroA-catalyzed reaction, PEP is broken down by formation of an oxocarbenium-ion or oxocarbenium-ion like structure. Circumstantial evidence such as formation of the S3P ketal, during the enzymatic and non-enzymatic reaction, formation of the oxocarbenium-ion complex during the non-enzymatic breakdown of THI, and inhibition of AroA by glyphosate all imply formation of an oxocarbenium-ion or oxocarbenium-ion like structure during the enzymatic and non-enzymatic reaction (*10, 12*). That is, that the C3 of PEP is protonated and the double bond is broken.

C-O bond cleavage





Non-enzymatic PEP breakdown can proceed via a P-O bond cleavage or a C-O cleavage (Fig. 28). To ensure that KIEs reflected on the formation of the

oxocarbenium ion and not P-O bond cleavage, PEP hydrolysis was done at pH below 0.

It was found that decreasing the pH increased the PEP hydrolysis via C-O bond cleavage. Also, the lack of formation of the singly or doubly deuterated PEP, when incubated under acidic conditions, showed that the oxocarbenium ion formed is not in equilibrium with PEP. This ensures that the KIEs measured on the acid-catalyzed breakdown of PEP reflect only the formation of the oxocarbenium ion.

4.4 Interpretation of KIEs on [3,3-²H₂]PEP on the Acid-Catalyzed Breakdown of PEP

KIEs on the acid-catalyzed breakdown of PEP using $[3,3-^{2}H_{2}]$ PEP were normal and significantly larger than the KIEs measured on the enzymatic reaction. The KIE of 1.180 ± 0.009 was measured and is comparable within expected values for deuterium secondary isotope effects. The ZPE contribution to the KIE is expected to be inverse. This is because the out-of-plane bending motion of the existing hydrons is restricted at the transition state as the incoming hydrogen approaches C3.

In the reactant PEP, C3 is sp^2 hybridized (Fig. 29). The planarity of C3 allows relatively free motion in the out-of-plane direction (Fig. 29, top right). In contrast, when C3 is protonated in the oxocarbenium ion, it becomes sp^3

hybridized (Fig. 29, bottom). The additional proton in the CH₃ group restricts the vibrational motion of the original two hydrons, leading to a tighter vibrational environment, and therefore an inverse contribution to the KIE (*63*). The size of the inverse ZPE contribution becomes larger as the transition state becomes more oxocarbenium ion-like and the amount of sp³ character of C3 increases. The effect would be larger if the transition state is more oxocarbenium ion-like. However, a normal isotope was measured, which can be explained by examining the isotope effect on the reaction co-ordinate itself.



Figure 29: Cartoon depicting the out-of plane bending movement of the existing hydrons of C3 in PEP compared with motion of hydrons of C3 in an oxocarbenium ion.

If the first irreversible step of the reaction is protonation of C3, then the reaction coordinate will include the motion of the incoming proton. As the proton

adds to C3 and C3 rehybridizes toward sp³, the original two hydrons must move as well. The motion of these hydrons becomes "coupled" to the approaching proton, and the out-of-plane bending motion of those two hydrons (toward the right in Fig. 30, centre) becomes part of the reaction coordinate (Fig. 30). As with any other harmonic oscillator, the frequency of the reaction coordinate depends on the mass of the atoms involved and is higher for lighter atoms. Thus, the reaction coordinate contribution to the KIE is always normal. KIEs on the reaction coordinate would be larger as the imaginary frequency becomes larger (63).

The KIE that will be observed in a reaction depends on the balance between the inverse contribution from the ZPE and the normal contribution from the reaction coordinate motion. In the acid-catalyzed hydrolysis of PEP, the contribution from reaction coordinate motion was larger than the inverse contribution, indicating that the proton is in the process of being transferred at the transition state (63).



Figure 30: Movement of C3 hydrons as part of the reaction coordinate motion during protonation of C3.

4.5 Summary of Transition State Features

For the enzymatic reaction, the ²H and ¹³C KIEs both indicate that a discrete oxocarbenium ion with a finite lifetime is being formed because they are similar to what would be expected for the equilibrium isotope effects between the reactant PEP and the THI. This indicates that protonation of C3 to form the oxocarbenium ion occurs during the first irreversible step of the reaction, and possibly a nucleophilic attack by O5' of S3P, or some non-chemical step (such as a conformational change). In contrast, the ²H KIEs for the acid-catalyzed reaction show that C3 protonation is the first irreversible step, with the proton being added to C3 still in-flight at the transition state.

5 CONCLUSIONS AND FUTURE WORK

Conditions were determined in order to measure KIEs accurately by whole molecule mass spectrometry for the AroA catalyzed reaction and the non-enzymatic hydrolysis of PEP. Isotopologues of PEP using ¹³C and ²H were synthesized and used to determine KIEs on the AroA-catalyzed reaction. $[3,3-^{2}H_{2}]$ PEP KIE was 0.985 and $[3-^{13}C]$ PEP KIE was 0.964. KIEs on the non-enzymatic acid-catalyzed hydrolysis of PEP using $[3,3-^{2}H_{2}]$ PEP was 1.180. This results suggests that the C3 carbon of PEP is fully protonated at the transition state for the non-enzymatic reaction.

By measuring KIEs at the remaining positions, the transition state structure of the acid-catalyzed hydrolysis of PEP can be deduced. Conditions have to be determined for the AroA reaction where reaction proceeds predominantly in the forward or reverse direction so that KIEs measured under those conditions can help deduce the transition state structure for the enzymatic reaction. Once the transition state structure is solved, analogs of the transition state can be used as potent inhibitors of AroA and can serve as a broad spectrum antibiotic.

6 **REFERENCES**

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