INHIBITION STUDIES OF CARBAMOYL PHOSPHATE SYNTHETASE FROM *Escherichia coli*

A Thesis

by

NEHA TRIPATHI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Chemistry

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Approved by:

Chair of Committee, Frank M. Raushel
Committee Members, Paul A. Lindahl
Gregory D. Reinhart
Head of Department, Emile A. Schweikert

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ABSTRACT

Inhibition Studies of Carbamoyl Phosphate Synthetase from *Escherichia coli.*

(December 2005)

Neha Tripathi, B.S.; M.S., Agra University, India

Chair of Advisory Committee: Dr. Frank M. Raushel

Carbamoyl phosphate synthetase (CPS) catalyzes the formation of carbamoyl phosphate (CP) from MgATP, bicarbonate, and glutamine. It has three active sites, one present on the small subunit and the two phosphorylation sites present on the large subunit. These two nucleotide binding sites are homologous. Six compounds were designed to mimic the reactive intermediate species carboxy phosphate, and product cabamoyl phosphate. The apparent K_i values calculated estimated the inhibitory strengths of these compounds. These plots were also utilized in identifying the linear inhibitors, nonlinear inhibitors and partial inhibitors. Inhibition patterns were obtained with these compounds using various assay formats. Partial inhibition displayed by phosphono formate for the full biosynthetic reaction can be utilized in support of the sequential mechanism for CPS.

DEDICATION

To my husband for his love, understanding and constant support;

To my grandmother,

To my parents for their endless efforts,

To my sisters and brother for uplifting my spirits,

To my son Abhishek for the joy he has brought in my life;

and to God.

ACKNOWLEDGMENTS

The completion of this thesis is a result of the contributions of many people. I would like to express my sincere thanks and gratitude to my advisor, Dr. Frank M. Raushel, for his constant guidance support and cooperation. I am also thankful to the members of my committee: Dr. Paul A. Lindahl and Dr. Gregory D. Reinhart.

Thanks to my former and current group members who always helped me and made my stay memorable. Special thanks to Alona P. Umali for synthesizing the compounds I have worked with. It was a pleasure working with you.

Thanks to my husband for the numerous trips he made from Houston to College Station. Thanks to my son, “little-warrior” Abhishek who has done so well after his illness. You have really inspired me.

Unwavering support of special friends, Sharath and Hema, who were with us through thick and thin gave me inner strength. Finally, thanks to all the friends in the Department of Chemistry, especially Poulomi, Aman, Susan and Hannan. Your encouragement and support made my journey a lot easier.

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CHAPTER I

INTRODUCTION

BACKGROUND

Carbamoyl phosphate synthetase (CPS) catalyzes the formation of carbamoyl phosphate (CP), an important intermediate in the arginine and pyrimidine nucleotide biosynthetic pathway (1, 3). It belongs to a family of enzymes called amidotransferases, like GMP synthetase, anthranilate synthase, imidazole glycerol phosphate synthase etc., which have a glutamine utilizing site with conserved cysteine, histidine and glutamate residues (2- 4). Ammonia is produced from the amide group of glutamine on the amidotransferase domain and subsequently utilized in the synthetase domain. The mechanism involves a nucleophilic attack by the thiolate anion of Cys^{269} on the carbonyl carbon of the carboxamide group. There is biochemical and structural evidence for the glutamyl thioester intermediate (5). Amidotransferases occur in a variety of pathways for biosynthesis of nitrogenous molecules including purine and pyrimidine bases, cofactors, aminosugars, amino acids and antibiotics. Thus amidotransferases provide an important but not exclusive route of incorporation of nitrogen in these molecules (2). Based on the conservation of the glutamine amidotransferase domain, two sub-families of amidotransferases, *pur* F (or F-type) and *trp* G (or G-type), are recognized. The sub-family of CPS is *trp* G (or G-type) amidotransferase because there is homology in the amidotransferase domains of CPS and anthranilate synthase, which is coded by the *trp* G gene. Other examples of G-type amidotransferases include aminodeoxychorismate synthase, formylglycinamide synthetase, CTP synthetase, GMP synthetase and imidazole glycerol phosphate synthase (2, 6). CPS is also a physiologically important enzyme because it helps the body get rid of the excess nitrogen by playing an important role in the urea cycle of a majority of terrestrial vertebrates (2, 3).

X-ray structure of CPS. CPS has been extensively studied for many years but its

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The format of this thesis follows the style of *Biochemistry*.

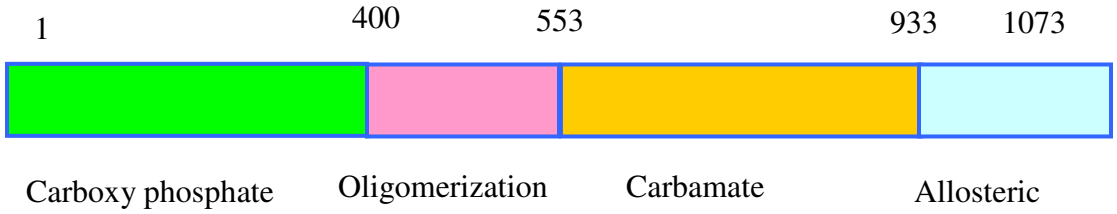
crystal structure was first solved in 1997 (7). The enzyme is an α, β-heterodimer which interconverts easily to an $(\alpha, \beta)_4$ octamer depending on the presence or absence of various effector molecules like ornithine and UMP (3). It consists of two subunits, a small glutamine-amidotransferase (GATase) (41,270 Da), encoded by the *car* A gene. It has a total of 382 amino acid residues (2). Hydrolysis of glutamine to form ammonia takes place here which is subsequently utilized by the large synthetase subunit (8). Mutations to Cys^{269} have revealed that this is the residue that confers the enzyme the capacity to utilize ammonia (2, 9). The crystal structure revealed that the small subunit is bilobal with the C-terminal domain predominantly having a 10-stranded mixed *β*-sheet similar to GMP Synthetase. The large synthetase subunit (117,700 Da) is encoded by the *car* B gene. It is composed of a total of 1073 amino acid residues. It houses the other two active sites and contains two ATP-grasp motifs (3, 7). The presence of two distinct ATP binding sites on the large subunit had earlier been established by steady state kinetics, site directed mutagenesis, and inactivation studies using 5'-p-fluorosulfonylbenzoyl adenosine (FSBA) (10). The fact that that the enzyme catalyzes two partial reactions also point towards catalysis at two ATP site (2). The mutation studies of the residues that are near or at the binding sites of the two nucleotide binding sites confirmed the function of two homologous halve of the large subunit. N-terminal half was postulated to be the bicarbonate phosphorylation site and the C-terminal half was suppose to be the carbamate phosphorylation site (11).

The crystal structure also revealed the presence of a molecular tunnel ~ 100 Å long spanning the entire molecule. The part of the tunnel ~ 45 Å long that connects the first active site to the second active site and is called the ammonia tunnel because it transfers ammonia from the small subunit to the phosphorylation site in the large subunit. The second half of the tunnel which is ~ 35Å connects the second and the third active sites and is called the carbamate tunnel because it channels the other reactive species carbamate.

Substrate channeling is an important feature of amidotransferases. It is thought to play an important role in metabolite regulation and cellular modulation of enzymatic activities (12).The molecular tunnel protects and channels the reactive intermediates from one active site to the next. The tunnel also enhances the catalytic efficiency by preventing

the diffusion of these intermediates into the bulk solvent. Thus substrate channeling is a common occurrence in enzymes with multiple catalytic sites (3).

Domain organization of CPS. The domain map of CPS is shown in Figure 1.1. It shows that the two homologous domains of the large subunit consist of residues 1-400 and 553-993. There is a 39 % identity and 25 % conservative replacements when two halves are compared (8). Residues from 400-553 are involved in the oligomerization of the enzyme. The allosteric domain is defined by residues 933-1073.



Scheme 1: Domain map of CPS (10).

Allosteric modulation of CPS. Apart from cradling the two nucleotide binding sites, the large subunit of CPS also has the allosteric domain. It is the site where the three allosteric effectors UMP, IMP, and ornithine bind (13, 14). CPS is an enzyme that plays a key role in the biosynthetic pathways of arginine and pyrimidine nucleotides. Hence UMP, which is an end product of the pyrimidine pathway, is a feedback inhibitor of the enzyme. Ornithine, however, is an activator of the enzyme whereas IMP can act both as an inhibitor or mild activator depending on assay conditions and temperature. UMP and the purine nucleotide IMP share the same binding site on the allosteric domain but ornithine has a separate binding site. UMP and IMP are competitive with each other while ornithine can be bound with either UMP or IMP. The binding sites are present at the end of C-terminal domain of the large subunit (14).

Reactions catalyzed by CPS. For the catalytic reaction, CPS needs two molecules of MgATP, HCO_3^- and glutamine as an ammonia source to form MgADP, inorganic phosphate (P_i), and carbamoyl phosphate. The full reaction is given in equation 1.

$2MgATP + HCO_3^- + glutamine + H_2O \rightarrow 2MgADP + Pi + glutamate + carbamoyl\ phosphate$ (eq.1)

Apart from catalyzing this full reaction, it also catalyzes three partial reactions. These are hydrolysis of glutamine, bicarbonate- dependent ATPase reaction, and ATP synthesis reaction utilizing ADP and carbamoyl phosphate. These reactions are shown in equations 2, 3 and 4 (15). In the absence of glutamine or ammonia, the synthesis and breakdown of carboxyphosphate is responsible for the ATPase activity of the enzyme (2).

$glutamine + H_2O \rightarrow \ glutamate + NH_3$ (eq.2)

$MgATP + H_2O \rightarrow \ MgADP + P_i$ (eq.3)

$MgADP + carbamoyl\ phosphate \rightarrow \ MgATP + NH_2CO_2^-$ (eq.4)

Kinetic mechanism of CPS. Raushel *et al* (1978) established the kinetic mechanism of the enzyme using initial velocity patterns and product inhibition studies (Figure 1.1). For the ammonia-dependent reaction they proposed a Ter-Uni-Uni-Ter ordered mechanism. MgATP, HCO_3^-, and ammonia bind to the active site of the enzyme followed by the release of P_i. The second ATP molecule binds before the release of MgADP, carbamoyl phosphate and MgADP in that order. When the nitrogen source is glutamine, the enzyme follows a two site mechanism in which glutamine binds to the small subunit of the enzyme in a random fashion. It is hydrolyzed to glutamate and ammonia which is transferred to the large subunit for the synthesis of CP (16).

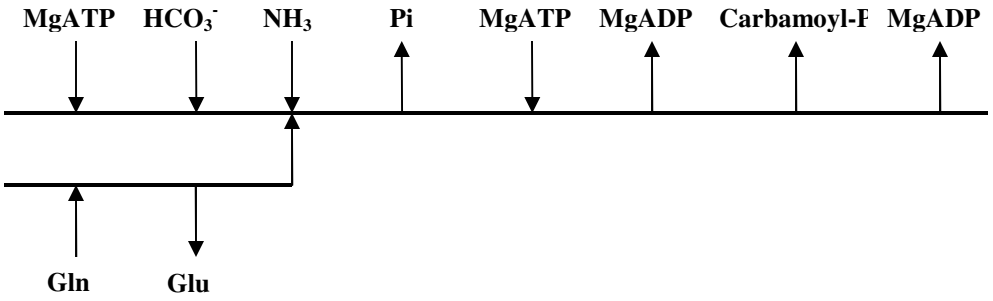


Figure 1.1: Representation of kinetic mechanism of carbamoyl phosphate (16).

Chemical mechanism of CPS. The mechanism for the *Escherichia coli* enzyme has been established by monitoring the partial reactions, intermediate trapping, and positional isotope exchange experiments (17). It involves four partial reactions and three reactive intermediates, carboxy phosphate, carbamate and ammonia (Figure 1.2) (18). The first intermediate, carboxy phosphate, is formed by phosphorylation of bicarbonate at the first ATP-binding site present on the N-terminal half of the large subunit. This is followed by a nucleophilic attack on this intermediate by ammonia to form the second intermediate, carbamate. At the second ATP-binding site, present on the C-terminal half of the large subunit, this intermediate undergoes phosphorylation to form carbamoyl phosphate (19).

SPECIFIC AIMS

Overall goal of this research. The specific aim of this research has been to distinguish between the two phosphorylation sites. It has been found that the N- and C-terminal halves of the large subunit are homologous. This situation has been proposed to arise from gene duplication and fusion of an ancestral kinase gene (8). The X-ray crystal structure has revealed an ATP-grasp fold present in both domains (7). These structurally similar domains phosphorylate bicarbonate and carbamate, but there is still some ambiguity regarding the roles of the two nucleotide binding site (10, 20). This research aims to develop inhibitors of the substrate, intermediate, and product mimics which can

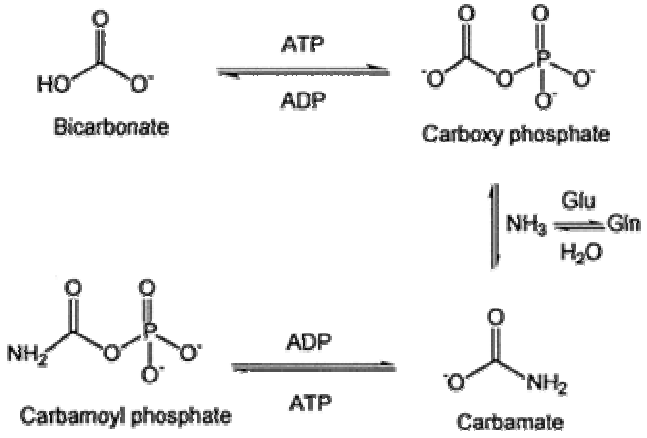


Figure1.2**:** Schematic representation of the mechanism of CPS showing three distinct intermediates (21).

serve as structural probes. Crystal structures complexed with these inhibitors could be used to obtain information regarding the binding of bicarbonate, carbamate and carbamoyl phosphate to the enzyme. Also any conformational changes which have not yet been seen in the enzyme upon binding of the substrate, reactive species and the product may be observed in the crystal structures (3).

Powers Lee and co workers have proposed a parallel or nucleotide switch mechanism for CPS. According to this mechanism, synthesis of carbamoyl phosphate takes place only within one nucleotide binding domain, carbamate is not transferred via the carbamate tunnel to the second phosphorylation site and the conformational changes occur at the second nucleotide binding site for which the energy is derived by the bicarbonate dependent hydrolysis of MgATP.

Isotopic oxygen exchange of ^{18}O and ^{16}O between the solvent water and [^{13}C] bicarbonate in the reaction in the absence of glutamine and no exchange in the presence of glutamine indicate that the carbamate actually is used in the synthesis of carbamoyl phosphate and is channeled through the tunnel. These results support the sequential mechanism of CPS (19). A crystal structure with one of the carbamoyl phosphate analogs bound to the C-terminal half of the large subunit would provide support for the actual migration of carbamate and further reinforce the sequential mechanism for the enzyme.

Earlier mutation studies have established residues essential in binding bicarbonate, carbamate, and carbamoyl phosphate (10, 22). A crystal structure of the enzyme co-crystallized with the inhibitors may enable us to identify residues involved in the binding and thus distinguish between the two phosphorylation sites in CPS. The kinetic studies with the analogs may be able to provide information about how the highly unstable intermediate species carboxy phosphate behaves before it reacts with ammonia and also the manner of binding of the second unstable species carbamate at the second phosphorylation site.

CHAPTER II

PRELIMINARY INHIBITION EXPERIMENTS WITH CARBOXY PHOSPHATE AND CARBAMOYL PHOSPHATE MIMICS

Carbamoyl phosphate synthetase is involved in the biosynthesis pathways of arginine and pyrimidine nucleotides. It catalyzes the synthesis of carbamoyl phosphate from ammonia, bicarbonate and ATP. Structurally it consists of two distinct subunits. The small subunit or the GAT domain which is 42,000 Da, produces ammonia from the amide of glutamine. The large subunit is 116, 000 Da and the synthesis of carbamoyl phosphate from bicarbonate, ammonia and ATP takes place on the C-terminal half of CPS. The N-terminal half produces a reactive intermediate carboxyphosphate. Amino acid sequence comparison has revealed that the two halves of the large subunit are homologous with 39 % identity (8). Despite this similarity the two nucleotide binding domains catalyze two different ATP-dependent partial reactions. The N-terminal half is responsible for the activation of bicarbonate through an ATP-dependent reaction. The C-terminal half of the subunit binds the second ATP molecule which phosphorylates carbamate leading to the synthesis of carbamoyl phosphate (11). In an attempt to distinguish the two nucleotide binding domains of the large subunit, six inhibitors were designed. These inhibitors would serve as structural probes of the reactive intermediate species carboxy phosphate and final product carbamoyl phosphate. One way of distinguishing the N-terminal and the C-terminal halves of the large subunit is by obtaining crystal structures complexed with one of these inhibitors. Information regarding the behavior of the inhibitor can be obtained from the preliminary experiments.

Dixon plots, a plot of $1/v$ versus inhibitor concentration [I] at some unsaturating substrate concentration [S], are employed to identify the type of inhibition and to determine the apparent K_i values (23). Preliminary experiments were performed with six compounds for three different assay formats. Unsaturating levels of substrates were fixed at their K_m levels. The structure of the six compounds, which are mimics of carboxy phosphate and carbamoyl phosphate are shown in scheme 2.

MATERIALS AND METHODS

Materials. All chemicals and coupling enzymes were purchased from Sigma and Aldrich. The inhibitors with the exception of phosphono acetic acid, which was purchased from Sigma, were synthesized by Dr. Alona P. Umali (Scheme 2).

Kinetic measurements. The rate of ADP formation was measured using a pyruvate kinase/lactate dehydrogenase coupling system (24). The reaction mixture contained 50 mM Hepes (pH 7.6), 5 mM $MgCl_2$, 100 mM KCl, 0.5 mM $KHCO_3$, 50 μM ATP, 10 mM ornithine, 1.0 mM phosphoenolpyruvate, 0.2 mM NADH, 20 units of pyruvate kinase and 30 units lactate dehydrogenase. The full reaction was performed using the pyruvate kinase/lactate dehydrogenase coupling system but it also contained 1.0 mM glutamine in the assay cocktail.

A hexokinase/glucose-6-phosphate dehydrogenase coupling system was utilized to monitor the rate of ATP formation from MgADP and carbamoyl phosphate. The assay cocktail contained 50 mM HEPES (pH 7.6), 100 mM KCl, 0.75 mM NAD^+, 1.0 mM glucose, 22 μM ADP, 2.0 mM carbamoyl phosphate, 20 units of hexokinase, and 10 units of glucose-6-phosphate dehydrogenase. The increase in absorbance was monitored at 340 nm (8, 11). All the assays were run at 25˚C and at pH 7.6. Data were fit to the following equations depending on whether the inhibitor was linear or displayed cooperative behavior. Data were fit to equation 5 to obtain the K_i values. Data for non-linear inhibitors were fit to equation 6. Data for phosphono formate were fit to equation 7 to obtain the K_i and α value.

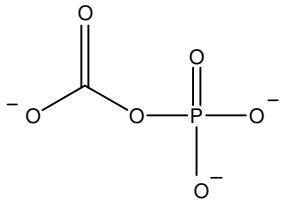
$$v = V / (1 + I / K_i) \quad \text{(eq.5)}$$

$$v = V / (1 + (I / K_i)^2) \quad \text{(eq.6)}$$

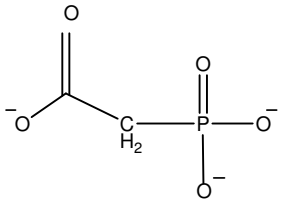
$$v = \alpha V / (1 + I / K_i) \quad \text{(eq.7)}$$

where V is the velocity when inhibitor concentration is zero, v is measured velocity, I is the inhibitor concentration, K_i is the inhibition constant and, α is the ratio of the velocities when inhibitor concentration is maximum and when inhibitor concentration is zero.

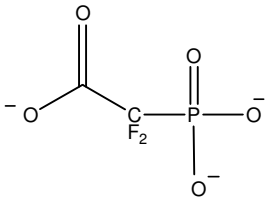
Scheme 2: Structures of carboxyphosphate and carbamoyl phosphate inhibitors



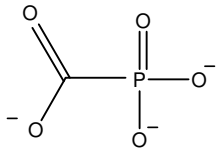
Carboxyphosphate



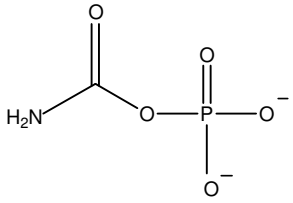
Phosphono acetic acid



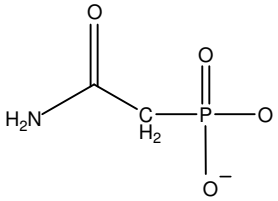
Difluoro phosphono acetic acid



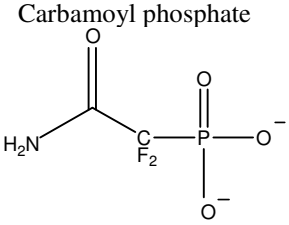
Phosphono formate



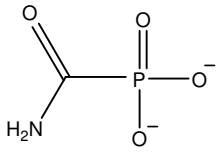
Carbamoyl phosphate



Phosphono acetamide



Difluoro phosphono acetamide



Phosphono formamide

In phosphono acetic acid and difluoro phosphono acetic acid, the bridging oxygen of carboxyphosphate has been replaced by $–CH_2$ and $–CF_2$ groups respectively. In phosphono formate the bridging oxygen of carboxyphosphate is not present. In phosphono acetamide and difluoro phosphono acetamide, the bridging oxygen of carbamoyl phosphate has been replaced by $–CH_2$ and $-CF_2$ groups respectively. In phosphono formamide O group of carbamoyl phosphate has been omitted.

RESULTS

Six compounds, which are mimics of carboxy phosphate and carbamoyl phosphate, were used as inhibitors of the wild type CPS. The inhibitory effects of these surrogates were gauged in preliminary experiments utilizing the two partial reactions and the full biosynthetic reaction catalyzed by CPS in the presence of the activator ornithine. The units of v and I^2 are mOD/min and mM^2 respectively.

Phosphono acetic acid. The preliminary survey of $1/v$ versus [I] of phosphono acetic acid from the partial forward reaction revealed that this carboxy phosphate analog showed linear inhibition. A plot of v versus [I] shown in Figure 2.1 was fit to equation 5. When the partial ATP synthesis reaction was monitored using phosphono acetic acid, it displayed non-linear inhibition (Figures 2.2 and 2.3). The data for the plot of $1/v$ versus I^2 were fit was fit to equation 6. It suggests cooperative behavior and binding to more than one enzyme form. Preliminary results for the full reaction of CPS with phosphono acetic acid are clearly a case of synergism as can be seen from the non-linear plot of $1/v$ versus inhibitor concentration [I] (Figure 2.4). A plot of $1/v$ versus I^2 was fit to equation 6 (Figure 2.5)

Difluoro phosphono acetic acid. For the partial forward reaction, this fluorinated carboxy phosphate analog behaved as a linear inhibitor when a plot of $1/v$ versus [I] was examined. A plot of v versus [I] was fit to equation 5 (Figure 2.6). For the partial back

reaction, the plot of 1/*v* versus inhibitor concentration [I] was not linear (Figure 2.7). A plot of 1/*v* versus I^2 was fit to equation 6 (Figure 2.8). The results suggests that the compound binds to multiple enzyme forms. For the full reaction, difluoro phosphono acetic acid behaved as a linear inhibitor as revealed by observing the data for 1/*v* versus [I]. A plot of *v* versus [I] was fit to equation 5 (Figure 2.9).

Phosphono formate. While observing the bicarbonate-dependent ATPase reaction, the1/*v* versus [I] plot showed phosphono formate to be a linear inhibitor. A plot of *v* versus [I] was fit to equation 5 (Figure 2.10). When monitoring the synthesis of ATP, this compound behaves as a non-linear inhibitor as seen in Figure 2.11. A plot of 1/*v* versus I^2 was fit to equation 6 (Figure 2.12). Interestingly for the full biosynthetic reaction this compound displayed only partial inhibition. The rate plateaus at a reduced velocity and does not reach zero even at a very high inhibitor concentrations. An α value of 0.4 was obtained from a fit of the data to equation 7. It is greater than zero is also indicative of partial inhibition (Figure 2.13).An initial estimate of the apparent K_i and the α values for these analogs with three different assay format are summarized in Table 2.1. These values were obtained by fitting the data to equations 5, 6 and 7.

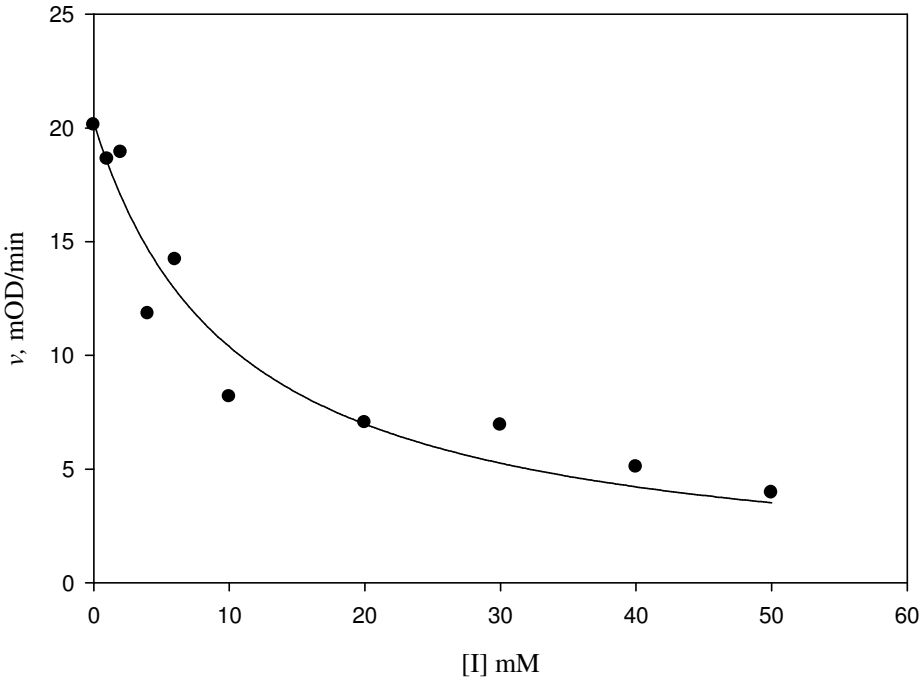


Figure 2.1: Plot of *v* versus [I] with phosphono acetic acid for the partial forward reaction from a fit of the data to equation 5.

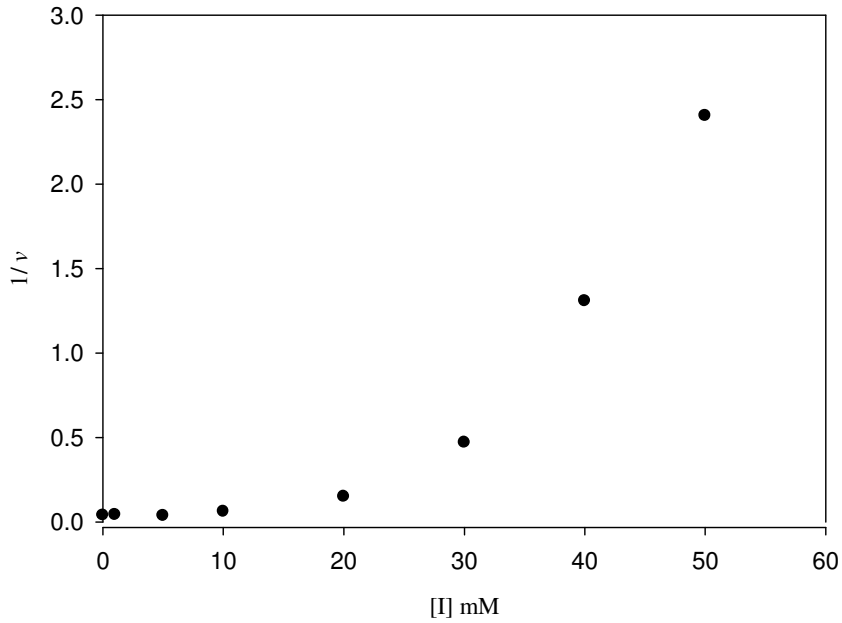


Figure 2.2: Plot of 1/*v* versus [I] with phosphono acetic acid for the partial back reaction.

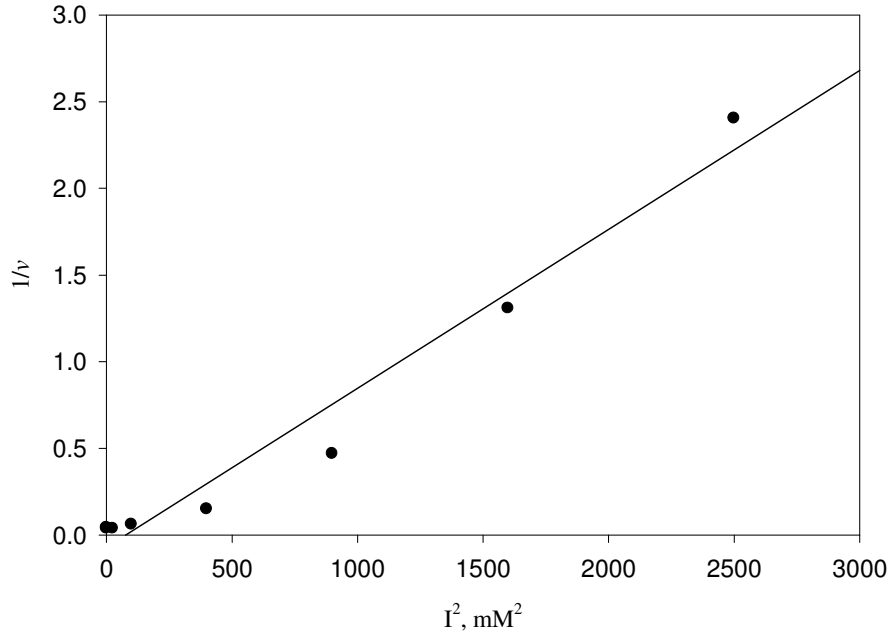


Figure 2.3: Plot of $1/v$ versus I^2 with phosphono acetic acid for the partial back reaction from a fit of the data to equation 6.

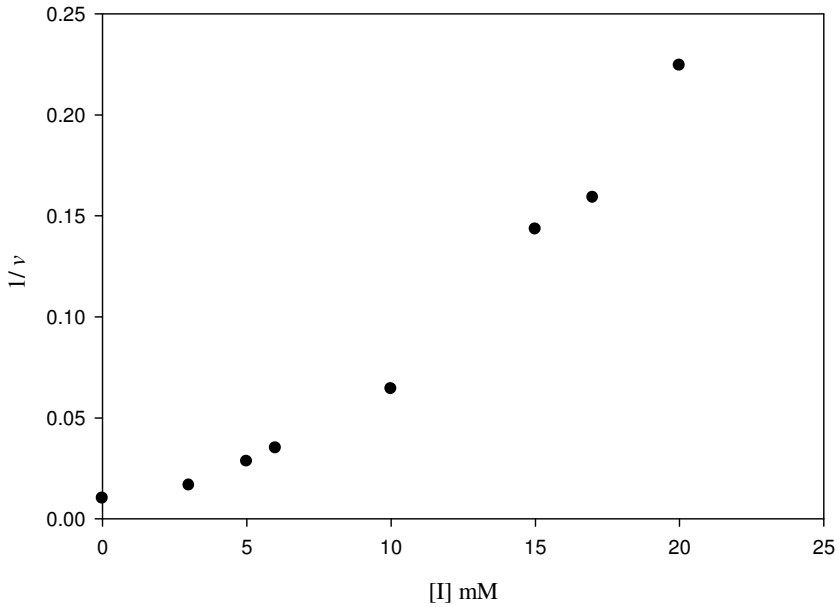


Figure 2.4: Plot of 1/*v* versus I with phosphono acetic acid for the full biosynthetic reaction.

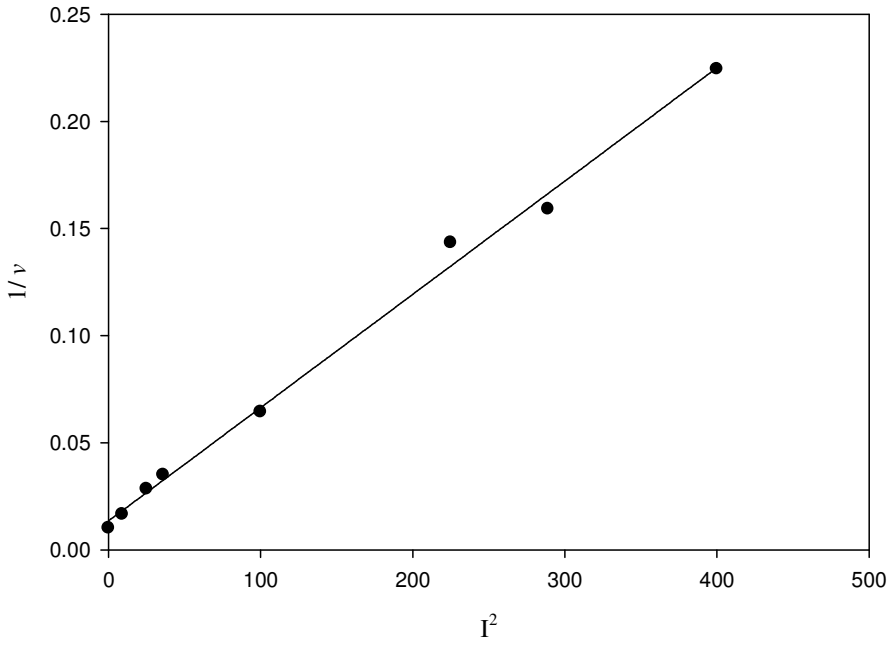


Figure 2.5: Plot of 1/*v* versus I^2 with phosphono acetic acid for the full biosynthetic reaction from a fit of the data to equation 6.

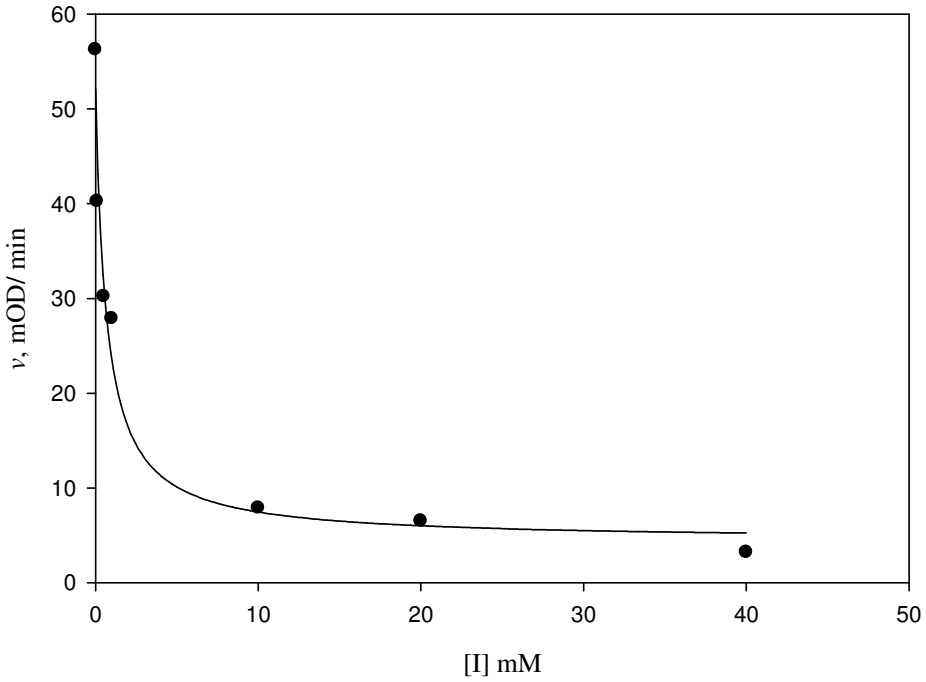


Figure 2.6: Plot of *v* versus [I] with difluoro phosphono acetic acid for the partial forward reaction from a fit of the data to equation 5

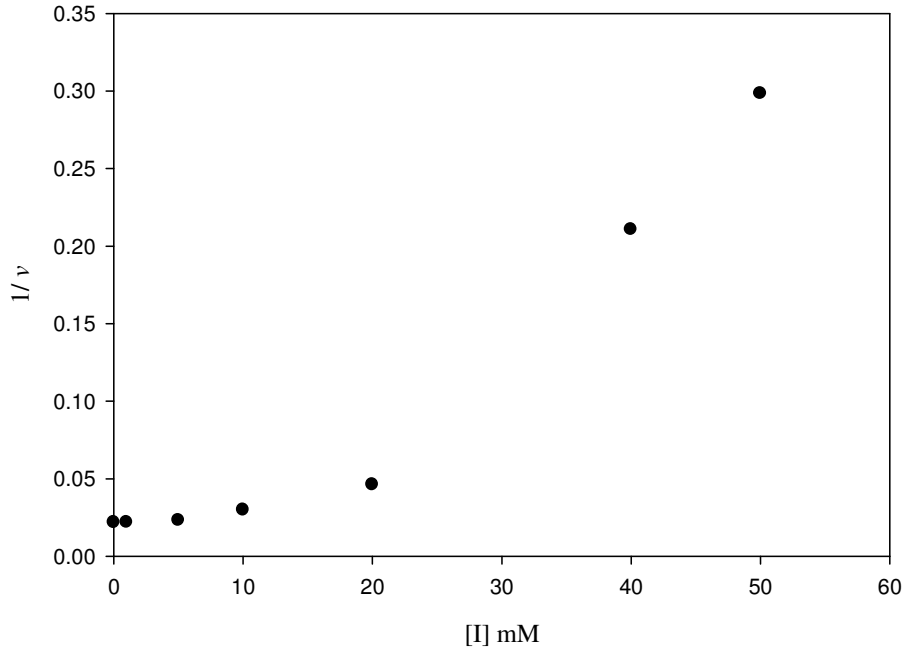


Figure 2.7: Plot of 1/*v* versus [I] with difluoro phosphono acetic acid for the partial back reaction.

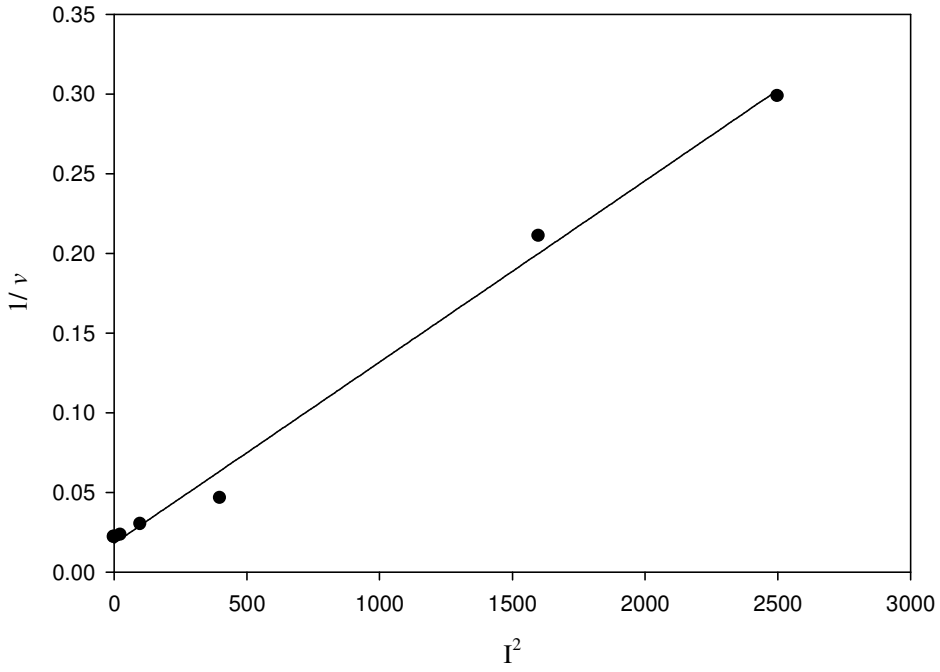


Figure 2.8: Plot of 1/*v* versus I^2 with difluoro phosphono acetic acid for the partial back reaction from a fit to the data to equation 6.

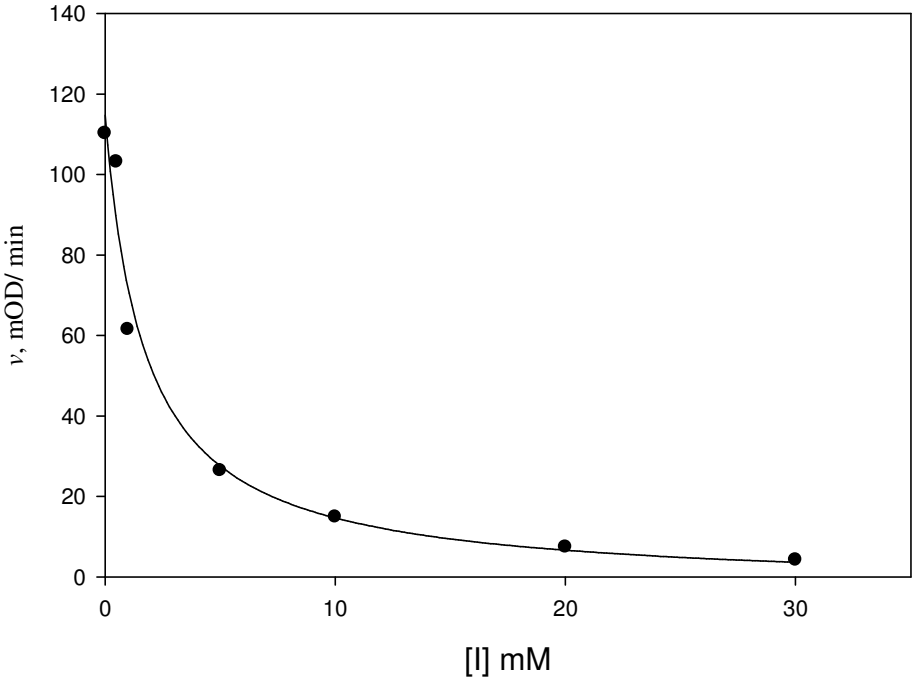


Figure 2.9: Plot of *v* versus [I] with difluoro phosphono acetic acid for the full biosynthetic reaction from a fit of the data to equation 5.

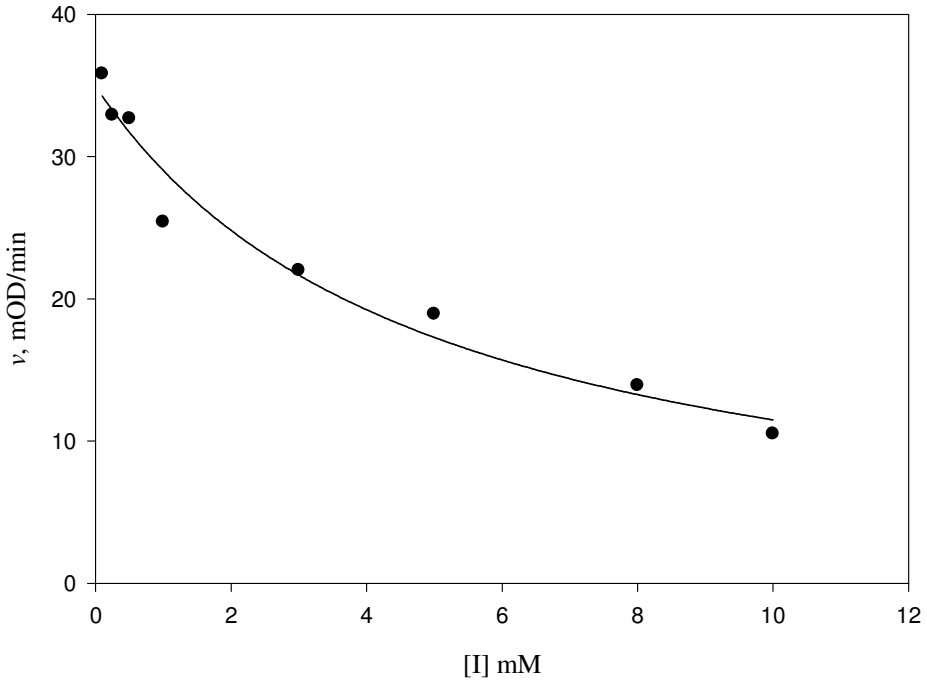


Figure 2.10: Plot of *v* versus [I] with phosphono formate for the partial forward reaction from a fit of the data to equation 5.

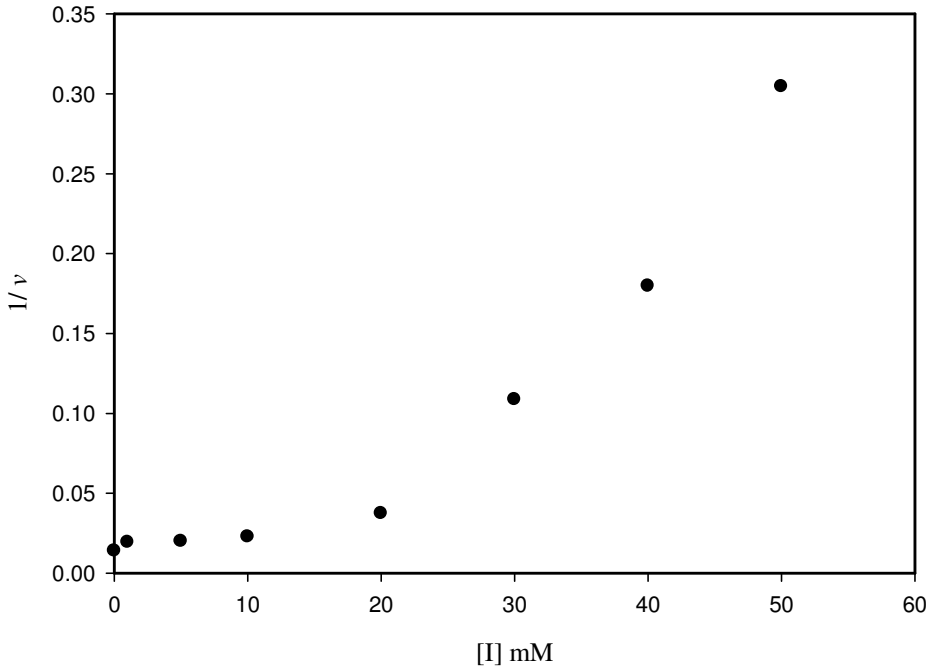


Figure 2.11: Plot of 1/*v* versus [I] with phosphono formate for the partial back reaction.

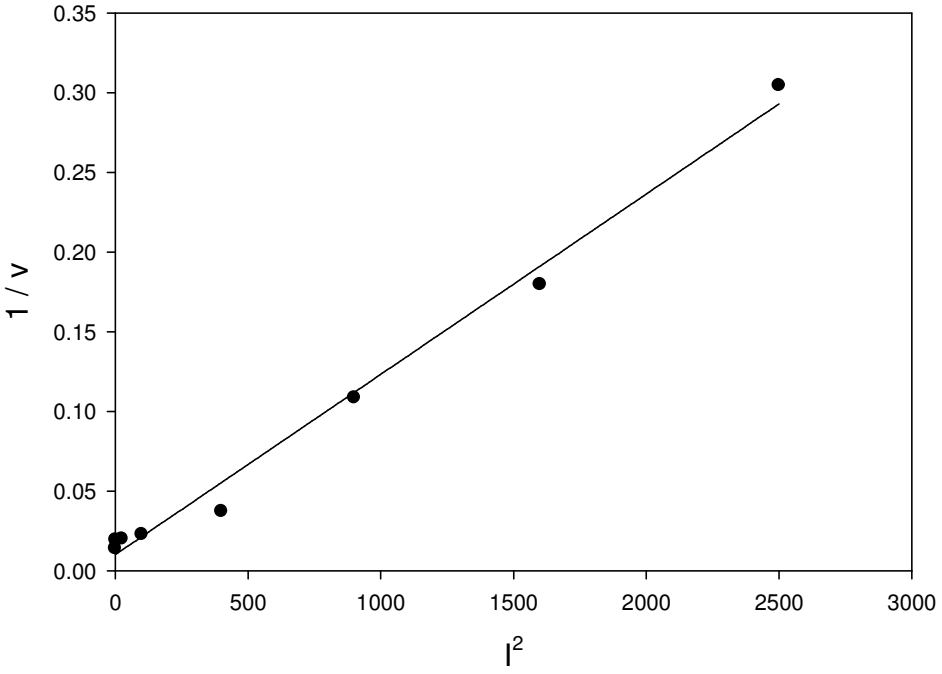


Figure 2.12: Plot of $1/v$ versus I^2 with phosphono formate for the partial back reaction from a fit of the data to equation 6.

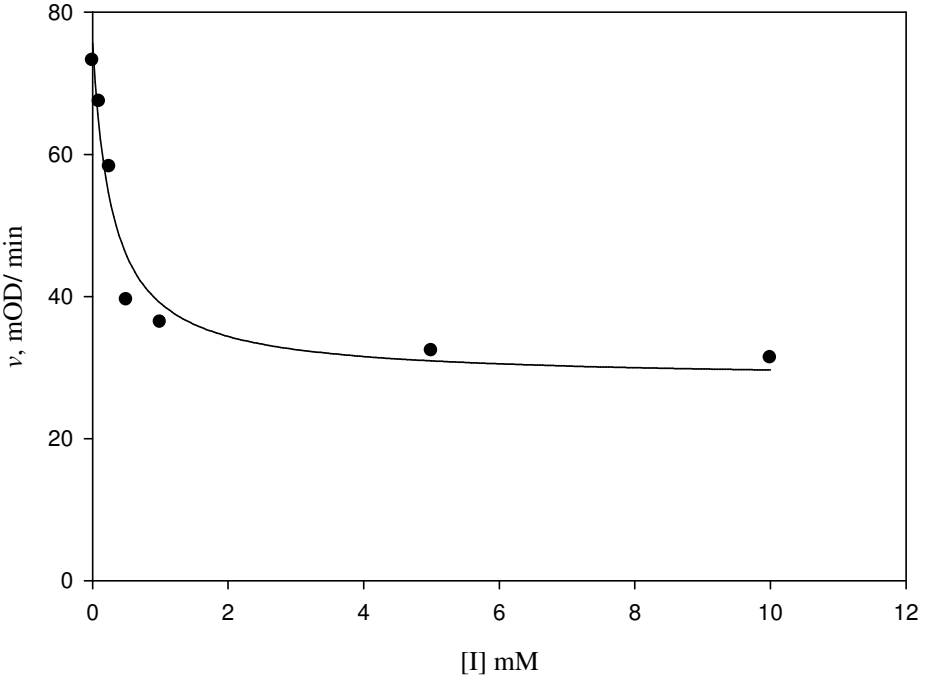


Figure 2.13: Plot of *v* versus [I] with phosphono formate for the full biosynthetic reaction from a fit of the data to equation 7.

Table 2.1: Apparent K_i values for the three carboxy phosphate analogs

Compounds	Reactions	$[HCO_3^-]$ mM	[ATP] µM	[Gln]. mM	[CP] mM	[ADP] µM	K_i mM	α	K_i^2 $(mM)^2$
Phosphono acetic acid	Partial forward	0.5	50				7.2 ± 3.0		
	Partial back				2.0	22			310 ± 120
	Full	0.5	50	1.0					380 ± 40
Difluoro phosphono acetic acid	Partial forward	0.5	50				0.7 ± 0.2		
	Partial back				2.0	22			700 ± 80
	Full	0.5	50	1.0			1.7 ± 0.7		
Phosphono formate	Partial forward	0.5	50				4.0 ± 2.0		
	Partial back				2.0	22			940 ± 230
	Full	0.5	50	1.0			0.3 ± 0 .01	0.4	

Phosphono acetamide. For the ATPase assay this carbamoyl phosphate mimcs displayed no inhibition as indicated from Figure 2.14. It shows no change in rate on increasing the inhibitor concentration. The highest inhibitor concentration used in this experiment was 50 mM. For the partial back reaction this carbamoyl phosphate mimic behaved as a linear inhibitor when a plot of 1/*v* versus [I] was examined. A plot of *v* versus [I] is given in Figure 2.15. When observing the hydrolysis of ATP in the presence of 1.0 mM glutamine, this compound caused no inhibition as evidenced by no change in rate on increasing the inhibitor concentration to 100 mM (Figure 2.16).

Difluoro phosphono acetamide. This compound does not inhibit CPS for the partial forward reaction. The rate of the enzymatic reaction remained essentially the same on increasing the inhibitor concentration as seen in Figure 2.17. The highest concentration of inhibitor used in experiment was 50 mM. While observing the partial back reaction, this compound behaves as a linear inhibitor. This was based on observation of the 1/*v* versus [I] plot. A plot of *v* versus [I] is given in Figure 2.18. This fluorinated analog of carbamoyl phosphate shows little inhibition for the full reaction, in the range of inhibitor concentration (40 mM) used in the experiment as seen in Figure 2.19.

Phosphono formamide. This analog of carbamoyl phosphate does not inhibit the partial forward reaction. Almost no change in rate is observed on increasing the inhibitor concentration as seen in Figure 2.20. For the partial back reaction, this compound behaves as a linear inhibitor. Figure 2.21 shows a plot of *v* versus [I] which was fit to equation 5. When monitoring the full biosynthetic reaction, this compound shows non-linear inhibition. Figure 2.22 shows a plot of 1/*v* versus [I] which clearly shows a non-linear trend. On plotting 1/*v* with a higher power of I (I^2) (Figure 2.23), the trend becomes linear. A summary of the apparent K_i values obtained by fitting the data to equation 5 and 7 is given in Table 2.2.

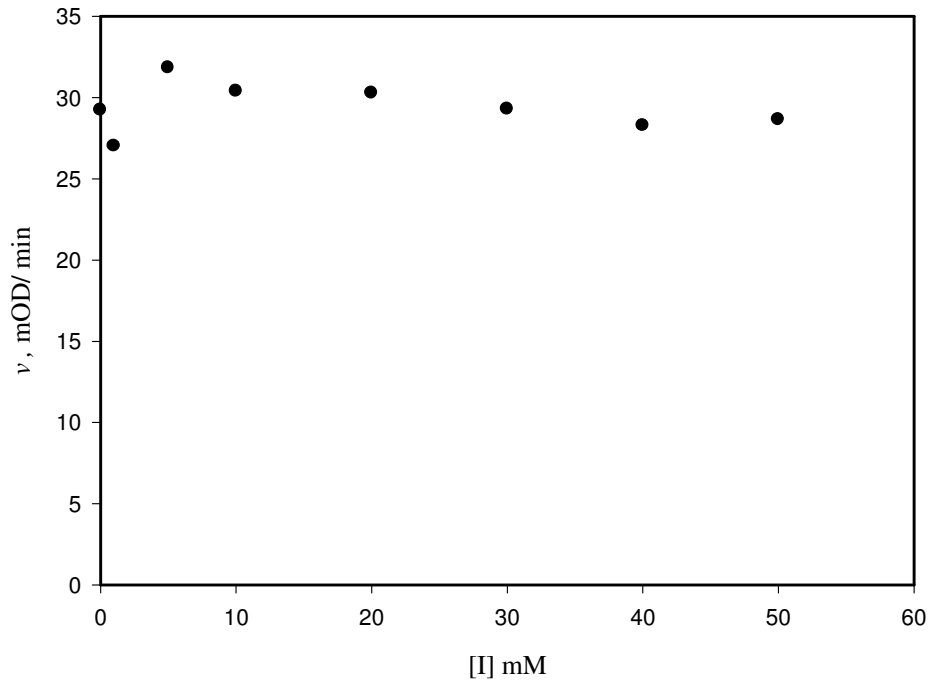


Figure 2.14: Plot of *v* versus [I] with phosphono acetamide for the partial forward reaction.

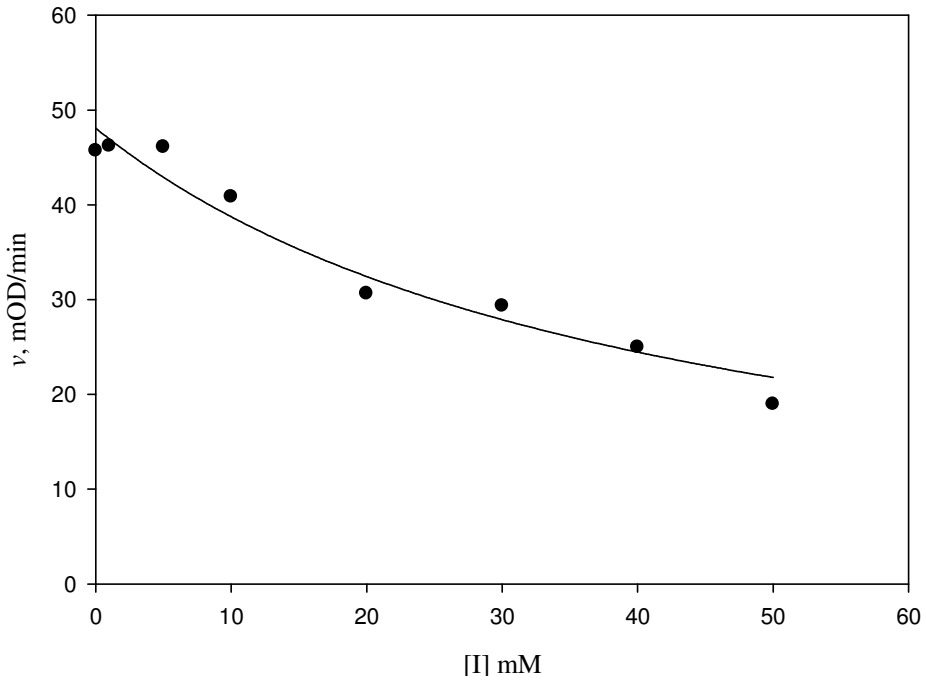


Figure 2.15: Plot of *v* versus [I] with phosphono acetamide for the partial back reaction fit of the data to equation 5.

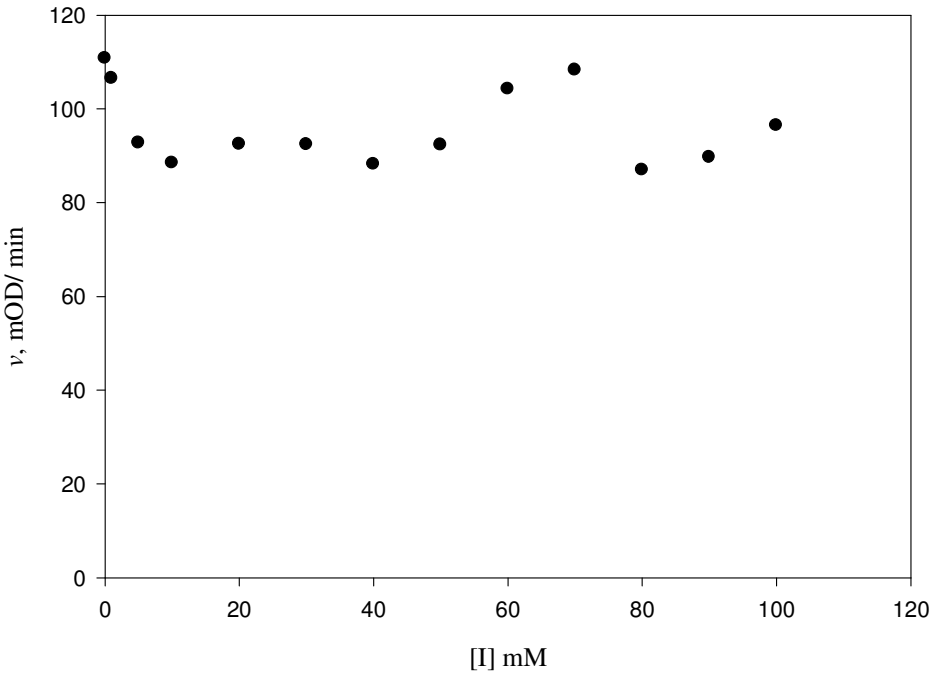


Figure 2.16: Plot of *v* versus [I] with phosphono acetamide for full biosynthetic reaction.

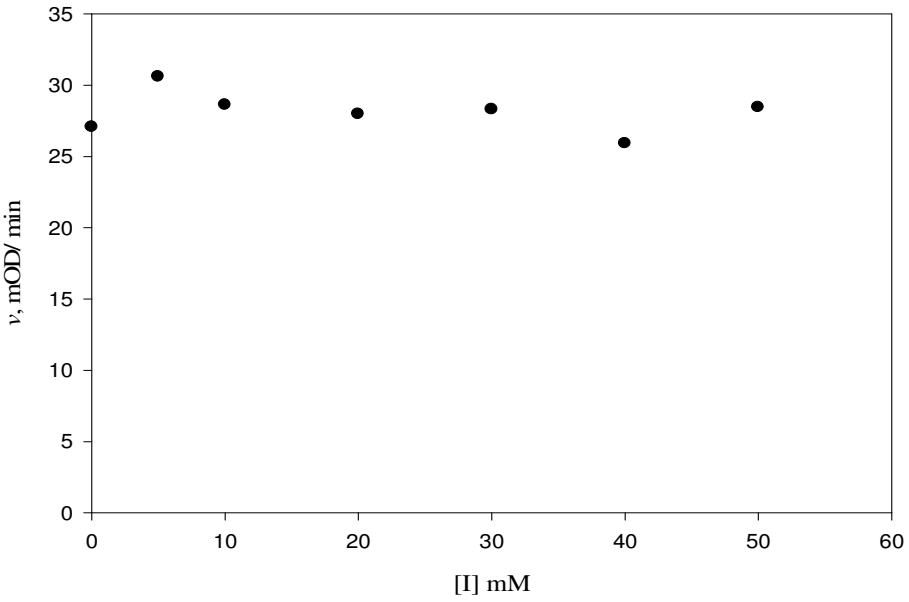


Figure 2.17: Plot of *v* versus [I] with difluoro phosphono acetamide for partial forward reaction.

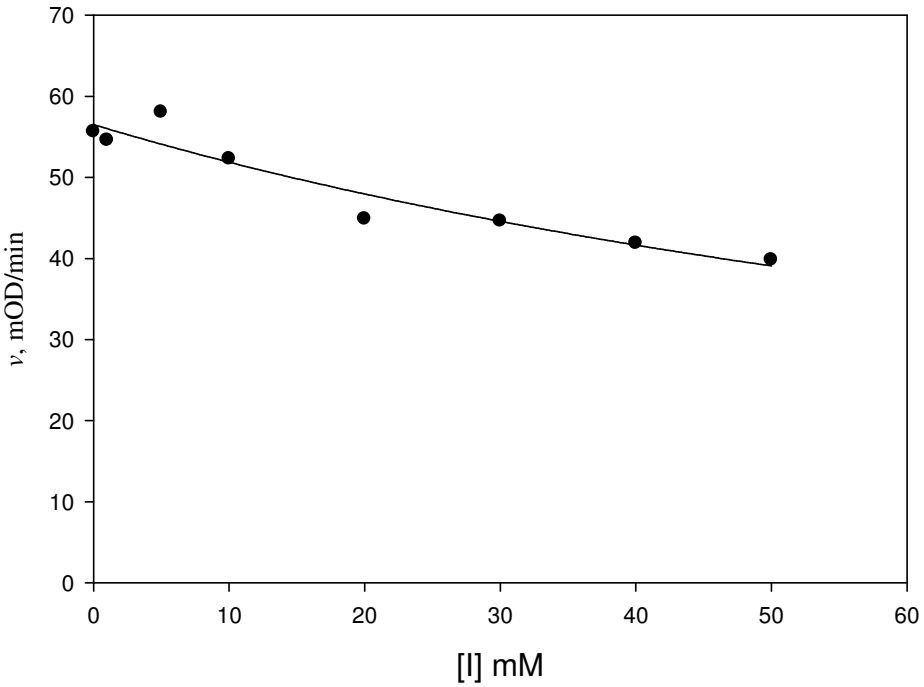


Figure 2.18: Plot of *v* versus [I] with difluoro phosphono acetamide for the partial back reaction from a fit of the data to equation 5.

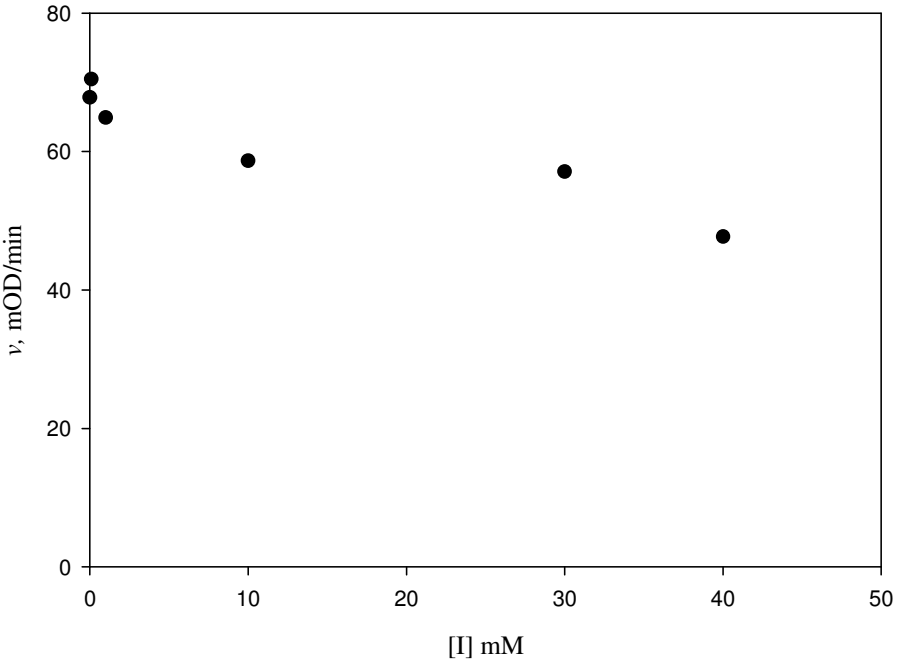


Figure 2.19: Plot of *v* versus [I] with difluoro phosphono acetamide for the full biosynthetic reaction.

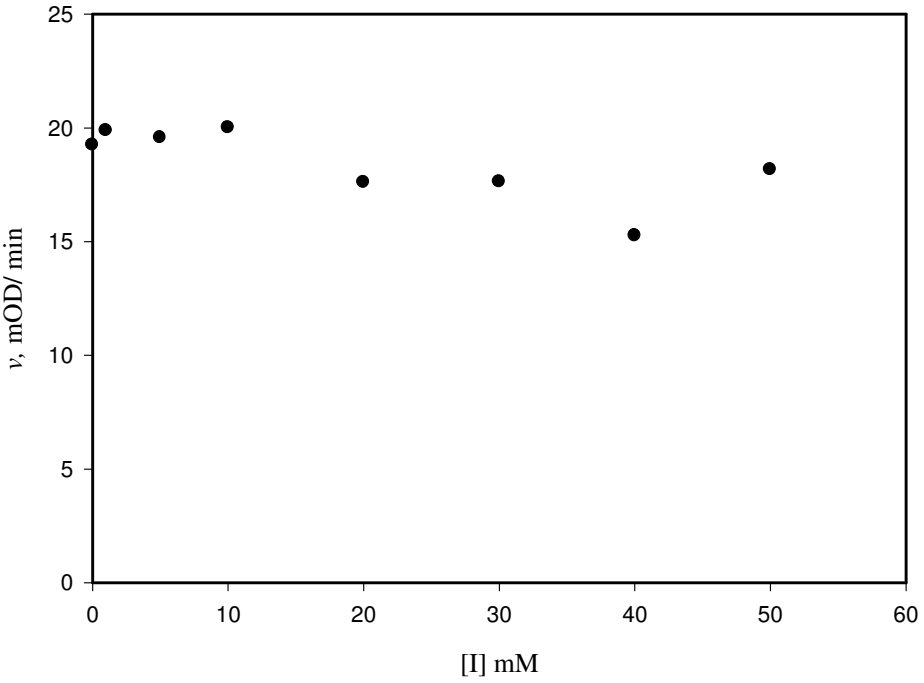


Figure 2.20: Plot of *v* versus [I] with phosphono formamide for the partial forward reaction.

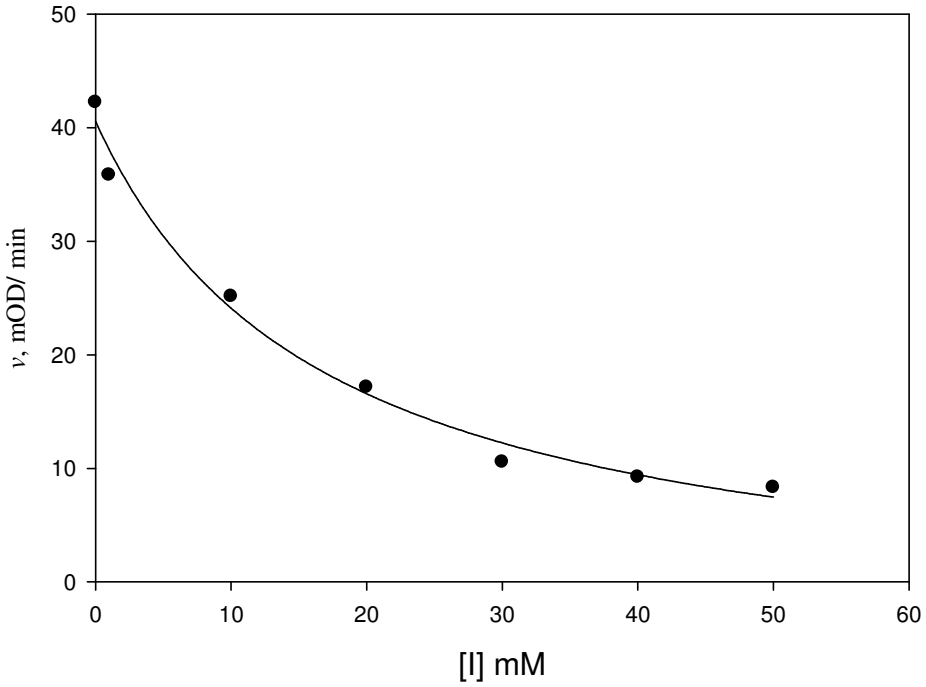


Figure 2.21: Plot of *v* versus [I] with phosphono formamide for the partial back reaction from a fit of the data to equation 5.

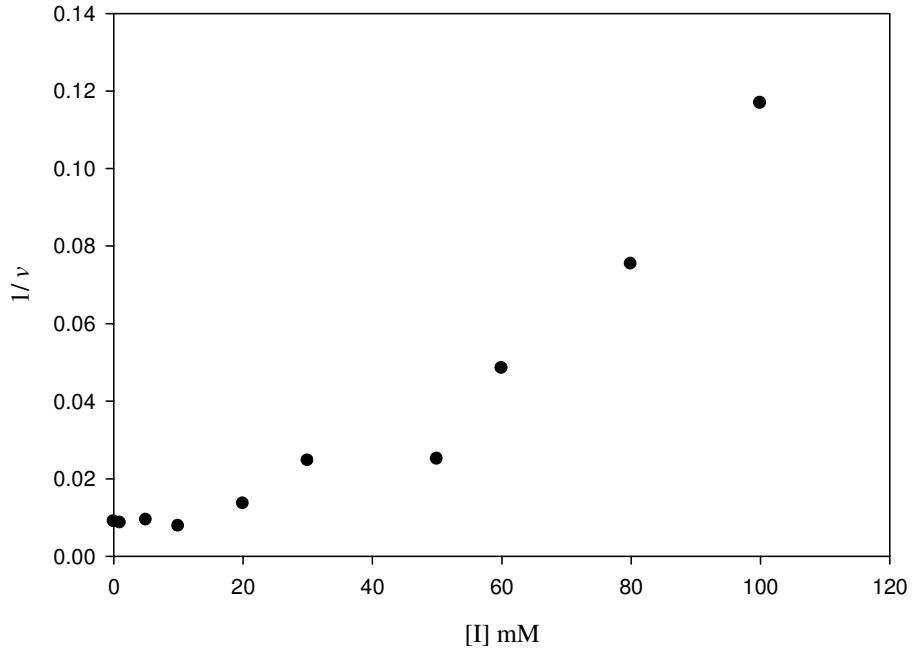


Figure 2.22: Plot of 1/*v* versus [I] with phosphono formamide for the full biosynthetic reaction.

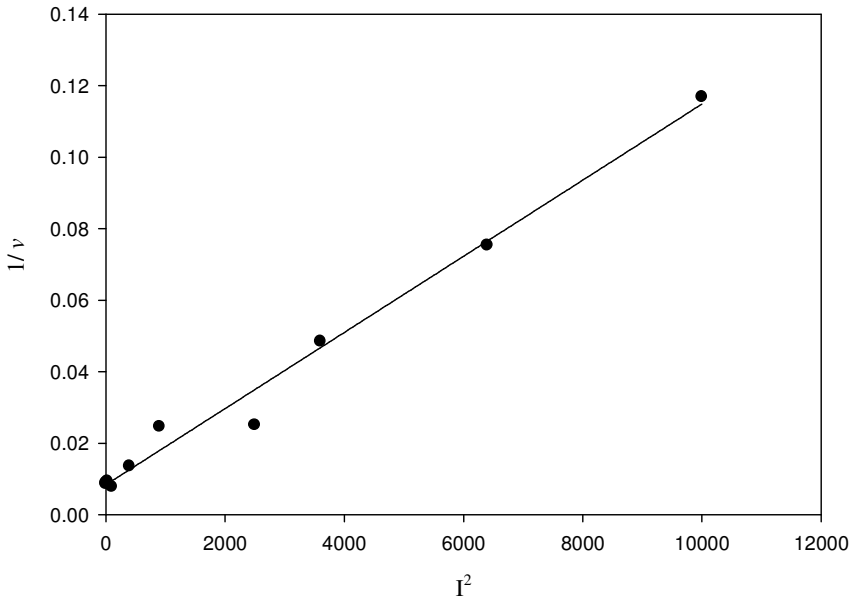


Figure 2.23: Plot of $1/v$ versus I^2 with phosphono formamide for the full biosynthetic reaction from a fit of the data to equation 6.

Table 2.2: Apparent K_i values for the three carbamoyl phosphate analogs

Compounds	Reactions	$[HCO_3^-]$ mM	[ATP] µM	[Gln]. mM	[CP] mM	[ADP] µM	K_i mM	K_i^2 $(mM)^2$
Phosphono acetamide	Partial forward	0.5	50					
	Partial back				2.0	22	41± 6.0	
	Full	0.5	50	1.0				
Difluoro phosphono acetamide	Partial forward	0.5	50					
	Partial back				2 .0	22	112 ± 17	
	Full	0.5	50	1.0				
Phosphono formamide	Partial forward	0.5	50					
	Partial back				2.0	22	13 ± 1.0	
	Full	0.5	50	1.0				3350± 310

DISCUSSION

Phosphono acetic acid. This compound is a structural mimic of carboxy phosphate. When monitoring the partial ATPase assay, it acts as a linear inhibitor for this reaction. Since only the N-terminal half of CPS, where the phosphorylation of bicarbonate takes place to form carboxy phosphate is involved in this reaction, it can be concluded that this mimic competes with bicarbonate and (or) ATP and binds to this phosphorylation site. For the partial back reaction which takes place on the second phosphorylation site or the C-terminal half of CPS, it is a non-linear inhibitor. It suggests that this inhibitor displays synergism and binds to multiple enzyme forms. Two molecules of the compound may bind to the phosphorylation site at the N-terminal half of the large subunit, one competing with bicarbonate and the other with ATP by virtue of its phosphate group or one molecule binding to the N-terminal phosphorylation site and the other to some other site other than the active site. In the glutamine-dependent ATPase reaction, this compound again displays synergism and binds at multiple sites. This compound possibly competes with bicarbonate and (or) ATP at the N-terminal half and the second ATP molecule at the C-terminal half of the large subunit.

Difluoro phosphono acetic acid. For the bicarbonate-dependent ATPase assay, this compound is a linear inhibitor and thus it can be concluded it binds to the N-terminal half of the large subunit where the phosphorylation of bicarbonate takes place to form carboxy phosphate, It may compete with bicarbonate and (or) ATP. While monitoring the partial back reaction which gives information about the C-terminal half of the large subunit where carbamoyl phosphate is synthesized, this compound acts as a non-linear inhibitor. This suggests a cooperative behavior which means that this compound is capable of binding to multiple forms of the enzyme. The binding could be at some sites other than the active site on the C-terminal half of the large subunit. While observing the glutamine-dependent ATPase reaction, difluoro phosphono acetic acid behaved as a linear inhibitor, thus suggesting that it binds only at a single nucleotide binding site. Since it behaves linearly for the ATPase assay, it means that it binds at the N-terminal half of the large subunit where carboxy phosphate is generated. On comparing the apparent K_i values of phosphono acetic acid and difluoro acetic acid, it can be concluded that the fluorinated analog is a better inhibitor for the partial forward reaction. This could

be because of the presence of $–CF_2$ group which is more electronegative than the $–CH_2$ group and hence serves as a better mimic of the bridging oxygen it is replacing in carboxyphosphate (Table 2.1).

Phosphono formate. This analog of carboxyphosphate behaves as a linear inhibitor suggesting that it binds at one active site. It competes with bicarbonate and (or) ATP. For the partial back reaction it displays cooperativity, meaning that it binds at multiple enzyme forms. Possibly two molecules bind at sites on the C-terminal half of the larges subunit but not necessarily on the active site. Interestingly for the full biosynthetic reaction it displays partial inhibition. It has an α value of 0.4. An alpha value of greater than zero means that inhibition is partial zero velocity is not possible even at higher concentrations of inhibitor. It suggests that the compound binds to one of the two ATP binding sites but not preventing the hydrolysis of ATP at the other nucleotide binding site leading to the synthesis of carbamoyl phosphate.

Phosphono acetamide. In the bicarbonate-dependent ATPase reaction this carbamoyl phosphate mimic causes negligible inhibition. On increasing the inhibitor concentration there is no apparent change in the rate of the enzymatic reaction. As the hydrolysis of ATP at the N-terminal half is being monitored in this reaction, it does not compete with any of the substrates. This compound does not inhibit the glutamine-dependent ATPase reaction also. On increasing the inhibitor concentration a negligible change in reaction rate is observed. Since it is a carbamoyl phosphate mimic it inhibits the partial back reaction in which one of the substrates is carbamoyl phosphate. It linearly inhibits the reaction and competes with carbamoyl phosphate and (or) with ADP since it also has the PO_3^- group. Thus it can be concluded that it binds to the phosphorylation site on the C-terminal half of the large subunit.

Difluoro phosphono acetamide. This compound is the fluorinated analog of carbamoyl phosphate. For the partial forward reaction it displays no inhibition. No decrease in the rate of the reaction is observed on increasing the inhibitor concentration. On observation of its inhibitory property in the partial back reaction, it is a linear inhibitor competing with carbamoyl phosphate and (or) ADP by virtue of its phosphate group. In the full reaction this compound shows negligible inhibition as the inhibitor

concentration is increased up to 40 mM. Thus this compound binds only to the phosphorylation site at the C-terminal half of the large subunit.

Phosphono formamide. This carbamoyl phosphate analog does not inhibit the partial forward reaction at the N-terminal half as there is essentially no change in rate on increasing the inhibitor concentration (50 mM). Thus no competition with either ATP or bicarbonate is observed. For the partial back reaction it binds to the C-terminal half as the diagnostic plot for it shows it to be a linear inhibitor. Here it possibly competes with carbamoyl phosphate and (or) ADP, for the active site. When monitoring the full biosynthetic reaction it is a non-linear inhibitor, which means it displays cooperativity and thus binds to multiple enzyme forms. Since this compound does not bind to the N-terminal half, both the binding sites for the full reaction are likely to be present on the large C-terminal half. Therefore two molecules of this compound could bind to the phosphorylation site here competing with carbamate and (or) ATP or one molecule could bind to the active site and the other to some other site on the enzyme.

Based on the preliminary experiments some general trends are observed for the acid phosphonates and the amide phosphonates. All three acid phosphonates which serve as structural mimics of carboxyphosphate are linear inhibitors of the partial forward reaction indicating that they compete with bicarbonate and (or) ATP. Difluoro phosphono acetic acid turns out to be the best inhibitor of the three with a K_i value of about 1.0 mM which is seven fold less than that of phosphono acetic acid which has a K_i value of 7.2 mM. Phosphono formate has K_i value of 4.0 mM which is about four fold higher than that of Difluoro phosphono acetic acid. This could be because the $–CF_2$ group is more electronegative and serves as better mimic of the bridging oxygen, which it replaces in carboxyphosphate.

In the partial back reaction all the three compounds display synergism which indicates that these acid phosphonates bind to more than one site on the C-terminal half of the large subunit where the reaction takes place. The binding sites could be, but not necessarily be the phosphorylation site. It is also possible that the compound binds to a site away from the active site.

For the full biosynthetic reaction, these compounds do not follow any general pattern and behave in a different manner for the three reactions. Phosphono acetic acid

displays cooperative behavior suggesting that it binds to multiple enzyme forms. It is possible that this particular compound binds to both the N-terminal and the C-terminal halves of the large subunit and. It could compete with bicarbonate and (or) ATP on the N-terminal half and also with the second ATP molecule at the C-terminal half. This can be said based on Figure 2.3 which does not quite linearize even when an I^2 term used. Difluoro phosphono acetic acid is a linear inhibitor for this reaction and binds to the phosphorylation site on the N-terminal half competing with bicarbonate and (or) ATP. It has a K_i value of about 2.0 mM which is two fold higher than its K_i value for the partial forward reaction. Phosphono formate shows partial inhibition. This compound binds to phosphorylation site on the N-terminal half at the same time not preventing the hydrolysis of ATP at the other phosphorylation site indicating that the synthesis of carbamoyl phosphate takes place at the C-terminal half and not on the N-terminal half. This does not support the nucleotide switch or parallel mechanism by Susan Powers Lee which proposes that the full biosynthetic reaction takes place on one single domain which alternate with each other (20). It does not have provision for the physical migration of carbamate, which is absolutely necessary for carbamoyl phosphate to be formed on the C-terminal half only.

The amide phosphonates which serve as carbamoyl phosphate surrogates do not bind at the N-terminal half because they are seen not to inhibit the partial forward reaction which takes place on the N-terminal half of the large subunit. This also supports the sequential mechanism for the enzyme where carbamoyl phosphate is synthesized exclusively on the C-terminal domain by the phosphorylation of carbamate. For the partial back reaction these compounds behave as linear inhibitors. Thus carbamoyl phosphate mimics bind to the C-terminal half of the large subunit. For the full biosynthetic reaction these compounds display little or no inhibition except phosphono formamide which displays synergism. The possible binding sites for this compound are present on the C-terminal half. One site could be present on the active site where it possibly competes with carbamate since its competition with ATP can be ruled out based on its behavior in the partial forward reaction. The second molecule may bind to a site away from the active site.

CHAPTER III

INHIBITION PATTERNS AND SUMMARY

Carbamoyl phosphate synthetase catalyzes the assembly of carbamoyl phosphate from two molecules of MgATP and a molecule of bicarbonate and glutamine. The reaction also produces glutamate, P_i and two molecules of MgADP. It consists of two polypeptide chains called the large and the small subunits. The small subunit (42 KDa) is known as the amidotransferase domain. It catalyzes the hydrolysis of glutamine to ammonia and glutamate which is then transported to the large subunit for the production of carbamoyl phosphate. The large subunit (118 KDa) is known as the synthetase domain. It can be visualized as two halves having an almost exact 2-fold rotational axis (7). This further corroborates the proposal that these two components arise from gene duplication and fusion of an ancestral protein (8). Despite the apparent homology in the sequences of the two domains of the large subunit, these domains perform two different functions. The aim of the inhibition studies is to assess why one domain phosphorylates bicarbonate and the other phosphorylates carbamate.

Inhibition patterns were obtained for the six carboxy phosphate and carbamoyl phosphate analogs were obtained. These compounds were tested for three different assay formats, bicarbonate-dependent ATPase glutamine-dependent ATPase, and the partial back reaction. For the first two assays, bicarbonate and ATP were the variable substrates. When bicarbonate was varied, the ATP concentration was kept constant at its K_m value (50 μM) and, alternatively, when ATP was varied, the bicarbonate concentration was kept constant at its K_m value (0.5 mM). For the partial back reaction ADP and carbamoyl phosphate were the variable substrates. When carbamoyl phosphate was varied the ADP concentration was fixed at 22 μM and when ADP was varied the carbamoyl phosphate concentration was kept at 2.0 mM.

MATERIALS AND METHODS

Materials. All chemicals and coupling enzymes were purchased from Sigma and Aldrich. The inhibitors except phosphono acetic acid which was purchased from Sigma were synthesized by Dr. Alona P. Umali. Their structures are given in Table 2.1.

Kinetic measurements. The rate of ADP formation was measured using a pyruvate kinase/lactate dehydrogenase coupling system (24). The reaction mixture contained 50 mM Hepes (pH 7.6), 5.0 mM $MgCl_2$, 100 mM KCl, 0.5 mM $KHCO_3$, 10 mM ornithine, 1.0 mM phosphoenolpyruvate, 0.2 mM NADH, 20 units of pyruvate kinase, 30 units lactate dehydrogenase and varying amounts of ATP. When varying amounts of bicarbonate were present, the ATP concentration was fixed at 50 µM. The full reaction was performed using the pyruvate kinase/lactate dehydrogenase coupling system but it also contained 1.0 mM glutamine in the assay cocktail.

The hexokinase/glucose-6-phosphate dehydrogenase coupling system was utilized to monitor the rate of ATP formation from MgADP and carbamoyl phosphate. The assay cocktail contained 50 mM HEPES (pH 7.6), 100 mM KCl, 0.75 mM NAD^+, 1.0 mM glucose, 20 units of hexokinase, and 10 units of glucose-6- phosphate dehydrogenase, and variable amounts of ADP and carbamoyl phosphate. The concentration of carbamoyl phosphate was fixed at 2.0 mM when ADP was varied and ADP was 22 µM when carbamoyl phosphate was varied. The increase in absorbance was monitored at 340 nm (8, 11). All the assays were run at 25˚C and at pH 7.6.

The K_{is} and K_{ii} values were determined by fitting the experimental data to equations 7-9. Equation 7 was for competitive inhibition, equation 8 for uncompetitive inhibition and equation 9 for noncompetitive inhibition

$$v = (V_m S)/(K(1+I/K_{is})+S) \quad \text{(eq.7)}$$

$$v = (V_m S)/(K+S(1+I/K_{ii})) \quad \text{(eq.8)}$$

$$v = (V_m S)/(K(1+I/K_{is}))+S(1+I/K_{ii}) \quad \text{(eq.9)}$$

In the equations v is the rate, V_m is maximal velocity, S is substrate concentration, I is the inhibitor concentration and K_{is} and K_{ii} are the inhibition constants.

RESULTS

Phosphono acetic acid. This compound mimics carboxy phosphate which is a highly unstable species produced within the N-terminal half of the large subunit. To determine how this compound behaves as an inhibitor, the partial forward reaction was monitored with variable bicarbonate and ATP. From the inhibition patterns it was evident that it is competitive against both bicarbonate and ATP (Figures 3.1 and 3.3). Figure 3.2 is a fit of the data to the equation 9. On comparing the errors in the K_i values, σ values and the variance Figure 3.1 was chosen over figure 3.2. The K_i values obtained are summarized in Table 3.1.

Difluoro phosphono acetic acid. This fluorinated analog competes with bicarbonate for the same enzyme form but when ATP was varied it was uncompetitive with it for both the partial forward and the full biosynthetic reaction (Figures 3.4, 3.6-3.8). This observation is consistent with the binding of this compound in a dead-end fashion to the E-ATP complex. Figures 3.5 is a fit of the data with difluoro phosphono acetic acid for the partial forward reaction against bicarbonate to equation 9 for noncompetitive inhibition. Figure 3.9 is a fit of the data for the full biosynthetic equation against ATP fit to equation 9 for noncompetitive inhibition. Competitive fit of Figure 3.4 was chosen over noncompetitive fit of Figure 3.5 and uncompetitive fit of Figure 3.8 over noncompetitive fit of Figure 3.9 based on the lower errors in the K_i values, σ values and the variance.

Phosphono formate. This compound behaved as a linear inhibitor for partial forward reaction in the preliminary experiment. When the inhibition patterns were obtained by varying bicarbonate and ATP, keeping the concentration of the other constant, in different experiments, phosphono formate was competitive against bicarbonate but noncompetitive against ATP as shown in Figures 3.10 and 3.12. These results suggest that the compound can form E.I and E.I.ATP complexes. Phosphono formate is competitive with both bicarbonate and ATP (Figures 3.13 and 3.15). This type of inhibition is consistent with the formation of an E.I complex that cannot bind either ATP or bicarbonate. Figure 3.11, 3.14 and 3.16 are fits of the data of phosphono formate for different assay formats to equation 9 for noncompetitive inhibition. The K_i values obtained for the carboxyphosphate analogs are summarized in Table 3.1.

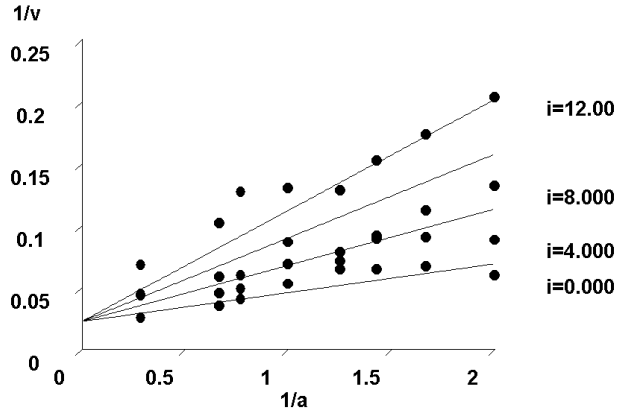


Figure 3.1: Competitive inhibition pattern with phosphono acetic acid against bicarbonate for the partial forward reaction from a fit of the data to equation 7.

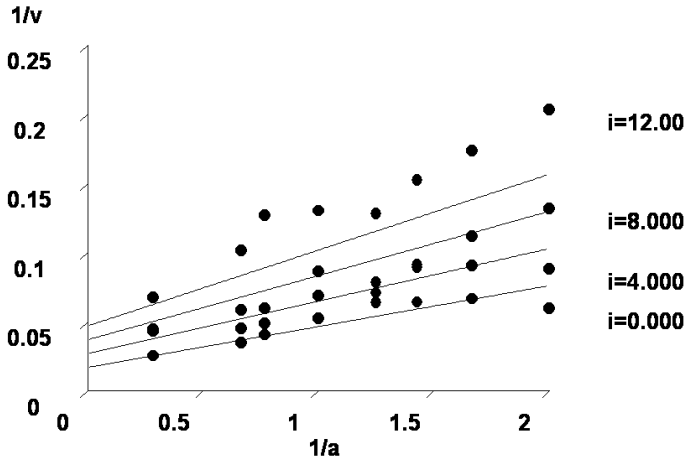


Figure 3.2: Noncompetitive fit with phosphono acetic acid against bicarbonate for the partial forward reaction from a fit of the data to equation 9.

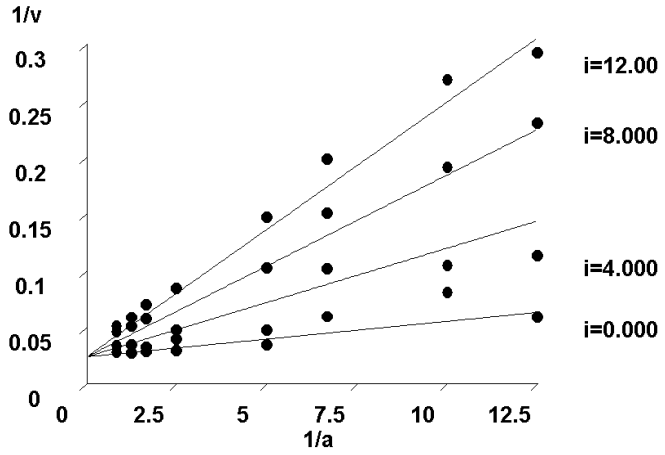


Figure 3.3: Competitive inhibition pattern with phosphono acetic acid for the partial forward reaction with variable ATP from a fit of the data to equation 7.

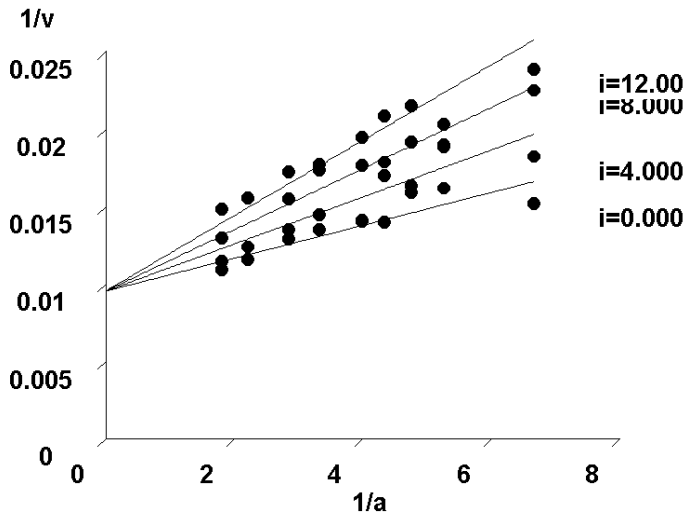


Figure 3.4: Inhibition pattern showing competitive pattern with difluoro phosphono acetic acid against bicarbonate for the partial forward reaction from a fit of the data to equation 7.

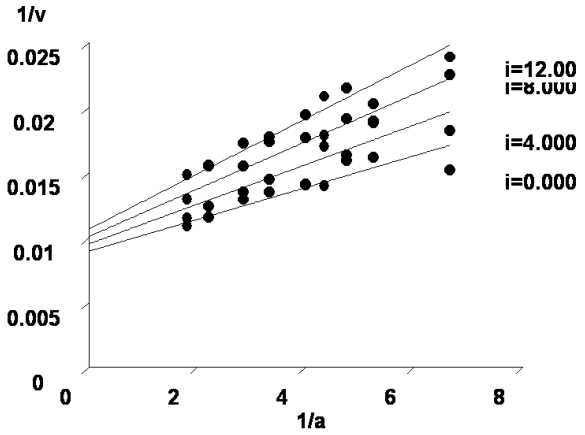


Figure 3.5: Inhibition pattern showing noncompetitive pattern with difluoro phosphono acetic acid against bicarbonate for the partial forward reaction from a fit of the data to equation 9.

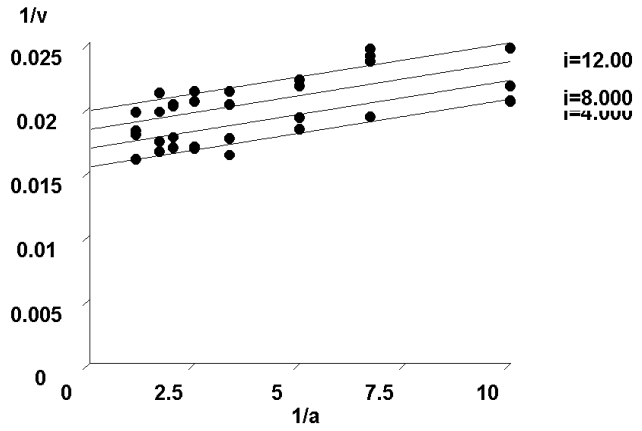


Figure 3.6: Uncompetitive inhibition pattern with difluoro phosphono acetic acid against ATP for the partial forward reaction from a fit of the data to equation 8.

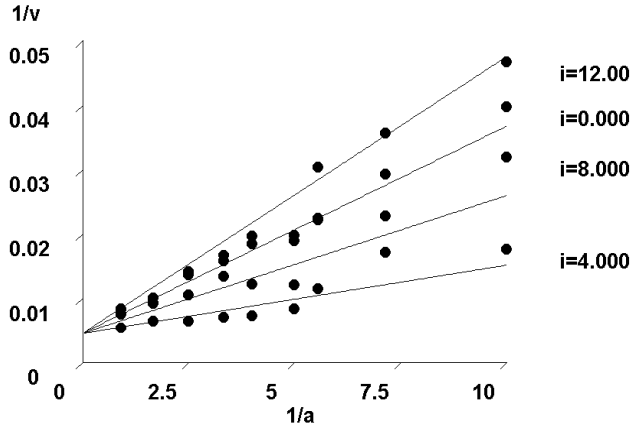


Figure 3.7: Competitive inhibition pattern with difluoro phosphono acetic acid against bicarbonate for the full biosynthetic reaction from a fit of the data to equation 7.

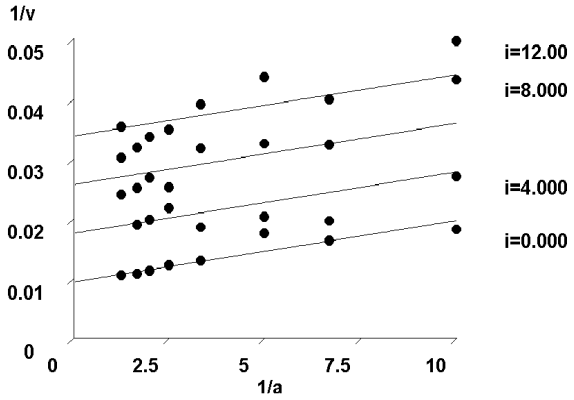


Figure 3.8: Uncompetitive inhibition pattern with difluoro phosphono acetic acid against ATP for the full biosynthetic reaction from a fit of the data to equation 8.

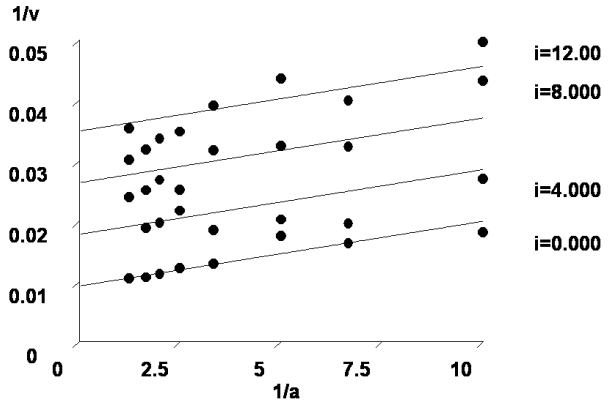


Figure 3.9: Noncompetitive inhibition pattern with difluoro phosphono acetic acid against ATP for the full biosynthetic reaction from a fit of the data to equation 9.

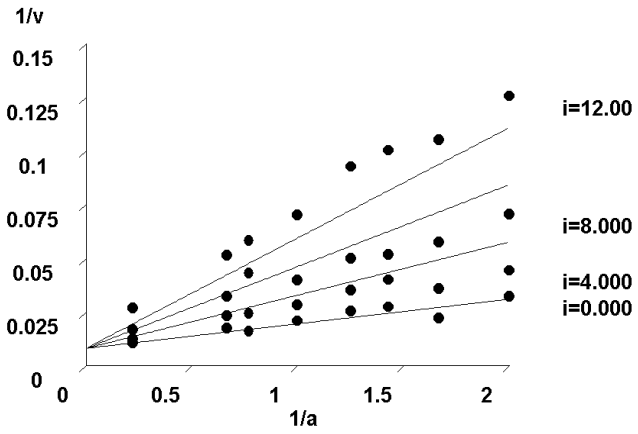


Figure 3.10: Competitive inhibition pattern with phosphono formate against bicarbonate for the partial ATPase reaction from a fit of the data to equation 7.

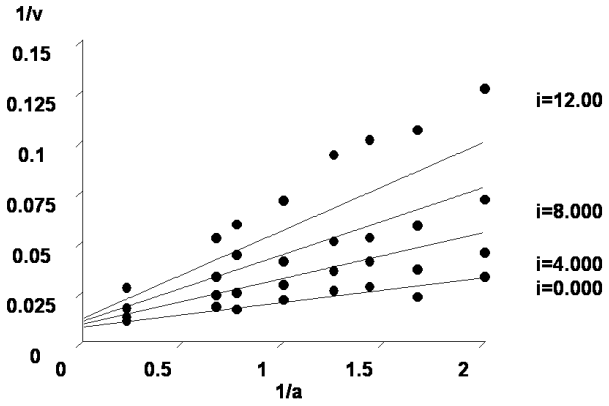


Figure 3.11: Noncompetitive inhibition pattern with phosphono formate against bicarbonate for the partial ATPase reaction from a fit of the data to equation 9.

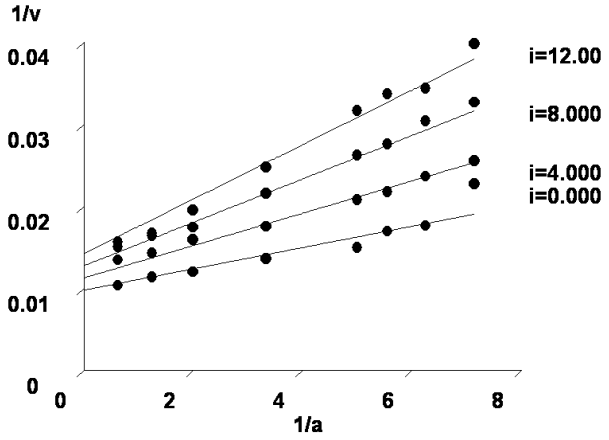


Figure 3.12: Noncompetitive inhibition pattern with phosphono formate against ATP for the partial forward reaction from a fit of the data to equation 9.

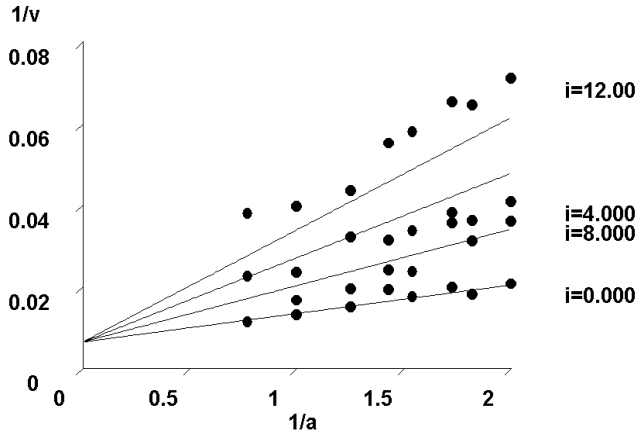


Figure 3.13: Competitive inhibition pattern with phosphono formate against bicarbonate for the full biosynthetic reaction from a fit of the data to equation 7.

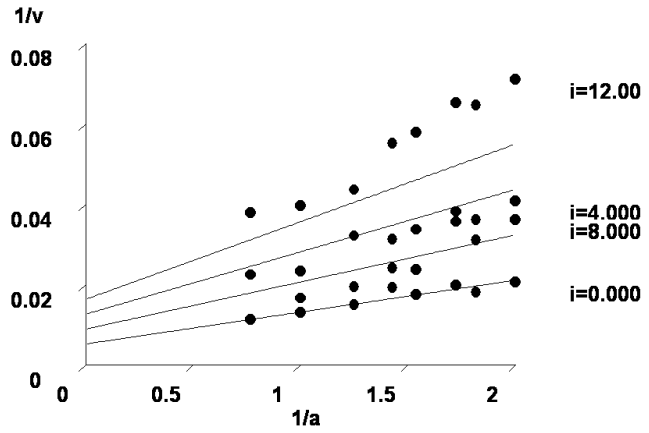


Figure 3.14: Noncompetitive inhibition pattern with phosphono formate against bicarbonate for the full biosynthetic reaction from a fit of the data to equation 9.

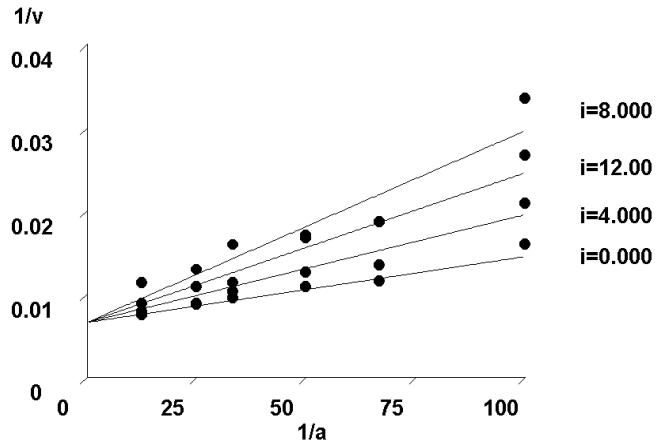


Figure 3.15: Competitive inhibition pattern with phosphono formate against ATP for the full reaction from a fit of the data to equation 7.

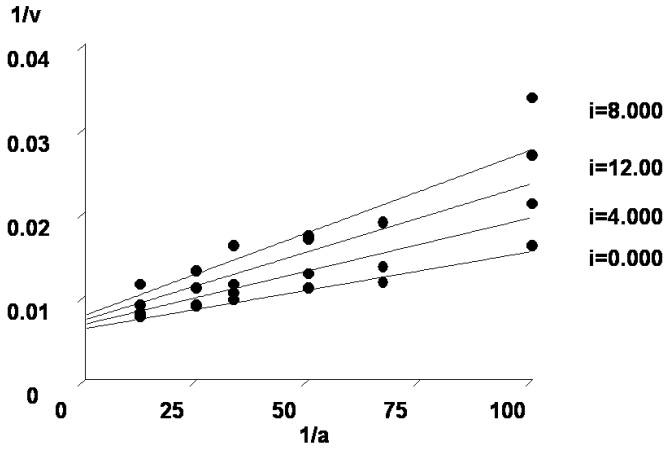


Figure 3.16: Noncompetitive inhibition pattern with phosphono formate against ATP for the full biosynthetic reaction from a fit of the data to equation 9.

Table 3.1: Apparent K_i values of phosphono acetic acid for partial forward reaction and difluoro phosphono acetic acid and phosphono formate for partial forward and full biosynthetic reaction

Compound	Reaction	Variable substrate	Fixed substrate	Inhibition type	K_{is} (mM)	K_{ii} (mM)
Phosphono acetic acid	Partial forward	HCO_3^-	50 µM ATP	C	4.2 ± 0.8	
		ATP	0.5 mM HCO_3^-	C	2.0 ± 0.3	
Difluoro phosphono acetic acid	Partial forward	HCO_3^-	50 µM ATP	C	9.3 ± 2.0	
		ATP	0.5 mM HCO_3^-	UC		42 ± 5.0
	Full	HCO_3^-	50 uM ATP	C	3.3 ± 0.6	
		ATP	0.5 mM	UC		5.0 ± 0.4
Phosphono formate	Partial forward	HCO_3^-	50 µM ATP	C	4.0 ± 0.4	
		ATP	0.5 mM HCO_3^-	NC	8.0 ± 1.4	26 ± 4.3
	Full	HCO_3^-	50 uM ATP	C	5.0 ± 0.7	
		ATP	0.5 mM	C	6.3 ± 1.2	

Phosphono acetamide. This carbamoyl phosphate analog inhibits the back reaction where carbamoyl phosphate is one of the substrates. It was noncompetitive against carbamoyl phosphate but competitive against ADP (Figures 3.17 and 3.18). Figure 3.19 is a fit of the data of this compound against ADP fit to equation 9 for noncompetitive inhibition. Competitive fit was chosen based on the lower error in the K_i value and lower σ value and variance.

Difluoro phosphono acetamide. This compound has the $–CF_2$ group replacing the bridging oxygen in carbamoyl phosphate. On monitoring the back reaction this compound turns out to be competitive against both carbamoyl phosphate and ADP (Figures 3.20 and 3.22). The data against carbamoyl phosphate seem to indicate cooperativity as opposed to the preliminary experiment which indicates that this compound is a linear inhibitor. Further experiments need to be done as it is hard to make any conclusions based on the experiment. The data against carbamoyl phosphate was also fit to equation 9 for noncompetitive inhibition as shown in Figure 3.21. This fit was eliminated based on higher errors in the K_i values and higher σ and variance.

Phosphono formamide. This compound is a mimic of carbamoyl phosphate. Partial back reaction which utilizes carbamoyl phosphate and ADP as substrates to synthesize ATP, takes place on the C-terminal half of the large subunit of CPS. Since it is structurally close to carbamoyl phosphate it is competitive against it and uncompetitive against ADP (Figure 3.23 and 3.25). Figure 3.24 represents the fit of the data against carbamoyl phosphate to equation 9 for noncompetitive inhibition. Fit to competitive inhibition was chosen based on lower error in the K_i value although fit to noncompetitive inhibition shows lower σ value and variance. Similarly the data against ADP was also fit to noncompetitive inhibition as shown in Figure 3.26. The uncompetitive fit was chosen based on the lower errors in K_i values even though it had slightly higher σ value and variance. Table 3.2 presents the K_i values for the compounds serving as carbamoyl phosphate analogs. The unit for 1/a in the plots is 1/ mM and the unit for v is mOD/min.

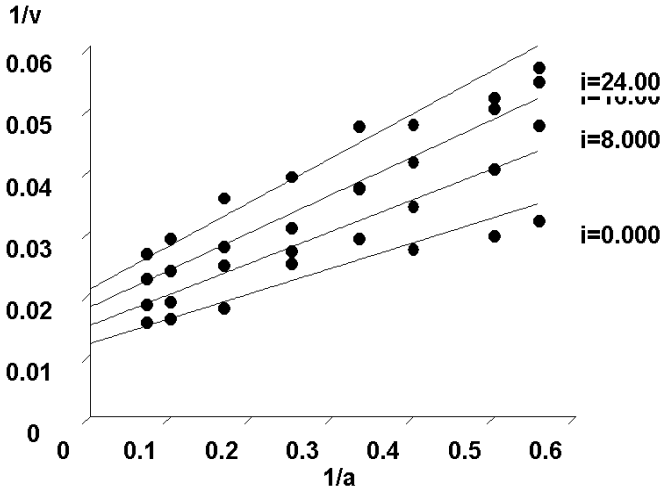


Figure 3.17: Noncompetitive inhibition pattern with phosphono acetamide against carbamoyl phosphate for the partial back reaction from a fit of the data to equation 9.

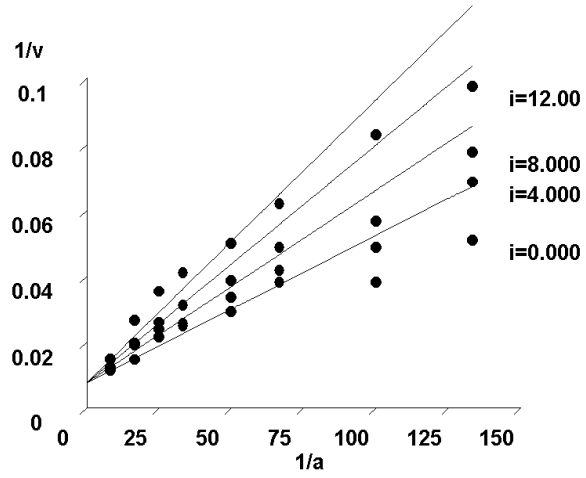


Figure 3.18: Competitive inhibition pattern with phosphono acetamide against ADP for the partial back reaction with from a fit of the data to equation 7.

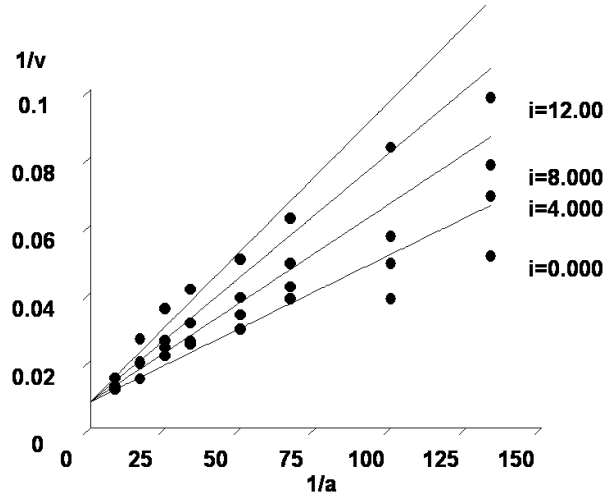


Figure 3.19: Noncompetitive inhibition pattern with phosphono acetamide against ADP for back reaction with from a fit of the data to equation 7.

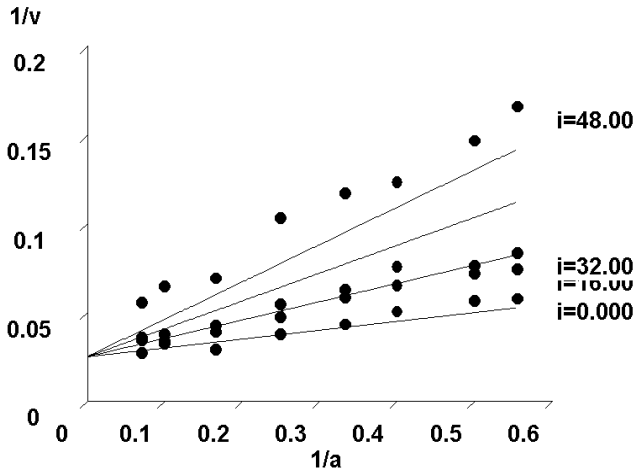


Figure 3.20: Competitive inhibition pattern with difluoro phosphono acetamide against carbamoyl phosphate for the partial back reaction from a fit of the data to equation 7.

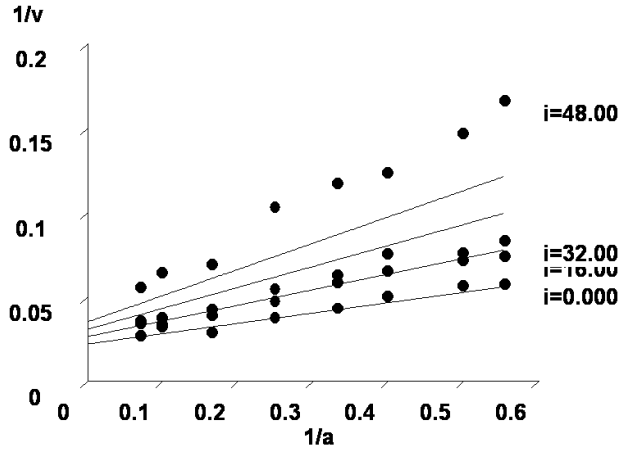


Figure 3.21: Noncompetitive inhibition pattern with difluoro phosphono acetamide against carbamoyl phosphate for the partial back reaction from a fit of the data to equation 9.

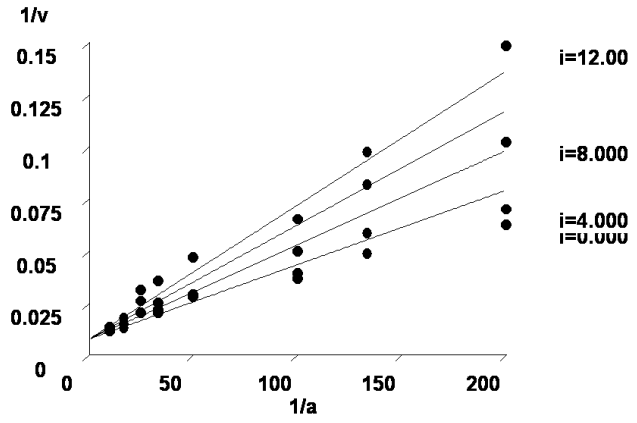


Figure 3.22: Competitive inhibition pattern with difluoro phosphono acetamide against ADP for the partial back reaction from a fit of the data to equation 7.

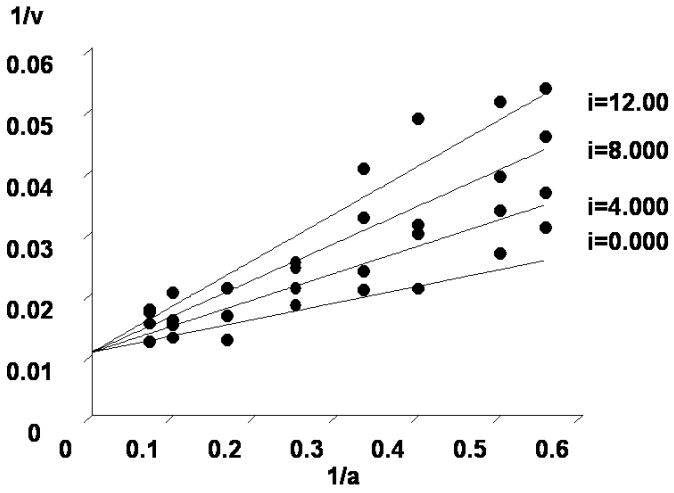


Figure 3.23: Competitive inhibition pattern with phosphono formamide against carbamoyl phosphate for the partial back reaction from a fit of the data to equation 7.

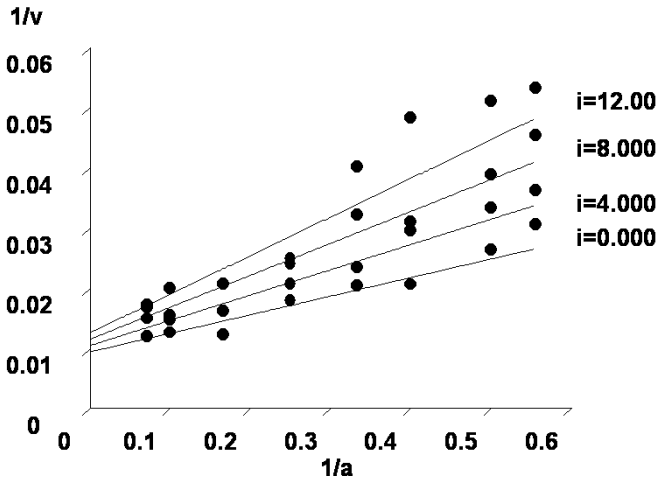


Figure 3.24: Noncompetitive inhibition pattern with phosphono formamide against carbamoyl phosphate for the partial back reaction from a fit of the data to equation 9.

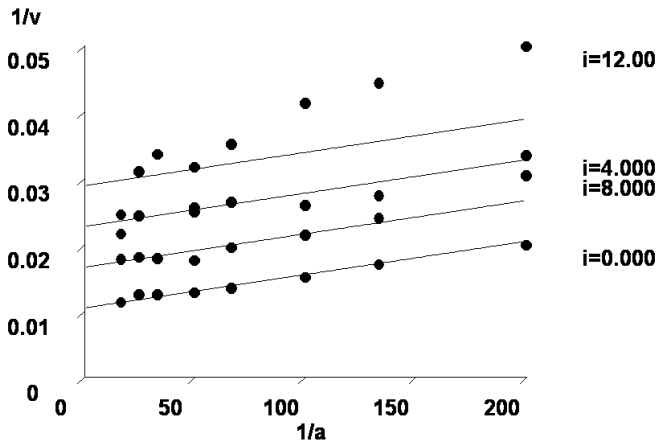


Figure 3.25: Uncompetitive inhibition pattern with phosphono formamide against ADP for the partial back reaction from a fit of the data to equation 8.

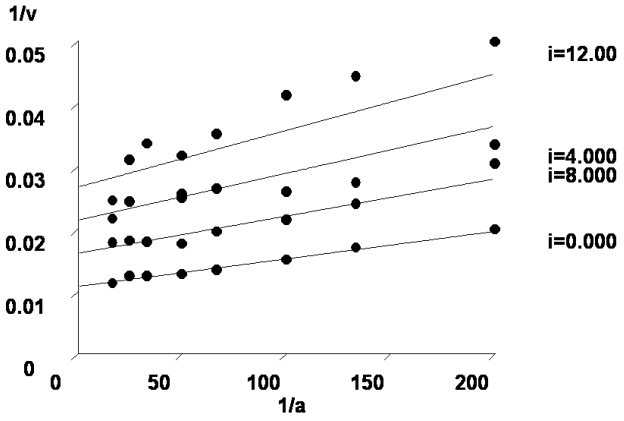


Figure 3.26: Noncompetitive inhibition pattern with phosphono formamide against ADP for the partial back reaction from a fit of the data to equation 9.

Table 3.2: Summary of apparent K_i values of phosphono acetamide difluoro phosphono acetamide and phosphono formamide for the partial back reaction

Compound	Reaction	Variable substrate	Fixed substrate	Inhibition type	K_{is} (mM)	K_{ii} (mM)
Phosphono acetamide	Partial back	CP	22 µM ADP	NC	33 ± 9.3	32 ± 6.0
		ADP	2.0 mM CP	C	13 ± 2.3	
Difluoro phosphono acetamide	Partial back	CP	22 µM	C	15 ± 3.0	
		ADP	2.0 mM	C	15 ± 3.0	
Phosphono formamide	Partial back	CP	22 µM	C	7.0 ± 1.2	
		ADP	2.0 mM	UC		7.0 ± 0.4

DISCUSSION

Bicarbonate- dependent ATPase assay. It is assumed that any changes to this assay would report on the part of the large subunit (N-terminal half) where this reaction takes place (17). It is within the ATP binding site that phosphorylation of bicarbonate takes place and the reactive intermediate species carboxy phosphate is formed. Thus phosphono acetic acid is competitive against bicarbonate and ATP with the K_{is} values of 4.2 mM and 2.0 mM respectively, when bicarbonate and ATP are varied. The competitive behavior against ATP could be due to the presence of the phosphate group on the compound. The fluorinated analog of carboxy phosphate, difluoro phosphono acetic acid was competitive against bicarbonate and uncompetitive against ATP. The K_{ii} and the K_{is} values are 9.3 mM and 42 mM, respectively. This compound thus competes for the same enzyme form with bicarbonate but since it is uncompetitive against ATP, it binds to the site available only after ATP is bound. Phosphono formate was competitive against bicarbonate and noncompetitive against ATP. This suggests that this compound competes with bicarbonate for the same enzyme form but since it is noncompetitive against ATP, it suggests that it may also bind to free enzyme to form a E.I. complex, thus causing a reduction in the catalytic rate. The three carbamoyl phosphate analogs did not inhibit the partial forward reaction so inhibition patterns were not obtained. This is because carbamoyl phosphate is utilized in the partial back reaction on the C-terminal half and not on the N-terminal half of the large subunit.

Partial back reaction. It is assumed here also that any inhibition of this reaction would shed light on the C-terminal part of the large subunit where it takes place (17). Inhibition patterns were obtained for three of the carbamoyl phosphate analogs. Phosphono formamide was uncompetitive (K_{ii} 7.0 mM) against ADP and competitive (K_{is} 7.0 mM) against carbamoyl phosphate. Competitive behavior suggests that it competes for the active site with carbamoyl phosphate but binds to the complex formed by the enzyme and ADP. Phosphono acetamide was competitive against ADP and noncompetitive against carbamoyl phosphate. This compound competed with ADP for the active site but since it showed noncompetitive behavior against carbamoyl phosphate, it would also to the ES complex to form the ESI complex. The fluorinated analog of carbamoyl phosphate, difluoro phosphono acetamide was competitive inhibition against

both carbamoyl phosphate and ADP .This compound would bind with the same enzyme form as both carbamoyl phosphate and ADP.

Glutamine- dependent ATPase assay. For the full reaction, the fluorinated analog of carboxy phosphate, difluoro phosphono acetic acid was competitive against bicarbonate and uncompetitive against ATP. Phosphono formate was competitive against both bicarbonate and ATP for the full reaction suggesting that this compound binds to the same enzyme form as bicarbonate and ATP.

SUMMARY

CPS is an enzyme which catalyzes the synthesis of carbamoyl phosphate. It has three active sites, one involved with the hydrolysis of glutamine to ammonia and glutamate is present on the small subunit and the two nucleotide binding sites where phosphorylation of bicarbonate and carbamate takes place to form highly unstable and reactive species carboxy phosphate and carbamate. The two domains are thus also known as the carboxy phosphate and carbamate domains. These domains are homologous as demonstrated by the studies of Nyunoya and Lusty (8), but there certainly is a difference in their functions as one phosphorylates bicarbonate and the other carbamate. Mutational analysis in the past has helped in identifying residues critical in catalysis but a crystal structure with an inhibitor may give further evidence as to the role of these residues. This inhibition study aims to differentiate the two phosphorylation sites.

Preliminary experiments were conducted for the two partial reactions and the full reaction with six inhibitors. For the partial forward reaction the two carboxy phosphate analogs behaved as linear inhibitors, suggesting that these bind only at one site. Further experiments where bicarbonate was varied and ATP was kept constant and when ATP was varied with constant bicarbonate concentration, phosphono acetic acid displayed competitive inhibition for both the experiments. It indicates that it competes for the active site with both ATP and bicarbonate and ATP. The K_i value was more (4.2 mM) when bicarbonate was varied than when ATP was varied (2.0 mM). Difluoro phosphono acetic acid displayed competitive inhibition when bicarbonate was varied and uncompetitive inhibition when ATP was varied. The K_i values were 9.3 mM and 42 mM respectively. Phosphono formate which is also a surrogate of carboxyphosphate also showed linear

competitive inhibition. Inhibition patterns obtained when bicarbonate and ATP were varied indicated that this compound was competitive against bicarbonate but noncompetitive against ATP. The other three compounds being analogs of carbamoyl phosphate did no inhibit the partial forward reaction. This is indicated by the plots of rate versus inhibitor concentration which showed negligible change in rate with increasing inhibitor concentration.

For the partial back reaction, all the three carboxy phosphate analogs behaved as non-linear inhibitors as indicated by the non-linear 1/ *v* versus inhibitor concentration [I] which were parabolic. Information regarding the different binding sites of an enzyme can be obtained by this multiple inhibition analysis. These non-linear plots require higher powers of [I] to linearize the data suggesting that there are more than two inhibitor binding sites (23). Preliminary experiments with the three carbamoyl phosphate analogs showed linear inhibition indicating they bind at one site only. Detailed experiments were conducted with variable carbamoyl phosphate and fixed ADP and alternatively with variable ADP and fixed carbamoyl phosphate. Phosphono formamide which lacks the bridging oxygen of carbamoyl phosphate, showed competitive inhibition with a K_{is} of 7.0 mM when CP was varied and uncompetitive inhibition with a K_{ii} of 7.0 mM when ADP was varied. It indicates that it competes for the same binding spot with carbamoyl phosphate due to the structural similarity but not with ADP. When ADP first binds the compound binds at a spot in the enzyme other then the active site causing possibly a change in the orientation of the active site. Phosphono acetamide was noncompetitive when carbamoyl phosphate was varied and competitive inhibition when ADP was varied. The fluorinated analog of carbamoyl phosphate, difluoro phosphono formamide showed competitive inhibition with both carbamoyl phosphate and ADP.

While observing the full biosynthetic reaction the carboxy phosphate analog phosphono acetic acid indicates non-linear inhibition. The fluorinated counterpart, difluoro phosphono acetic acid however exhibits linear inhibition. It follows then that linear inhibitor binds only at one site while the other binds possibly at multiple binding sites. It has K_{is} and K_{ii} values of 3.3 mM and 5.0 mM when bicarbonate and ATP are varied respectively. Partial inhibition was seen when phosphono formate was utilized as the structural mimic of carboxyphosphate in the preliminary experiment for the full biosynthetic reaction. It was competitive against both bicarbonate and ATP when inhibition patterns were obtained. Carbamoyl phosphate mimics phosphono formamide was a non-linear inhibitor displaying synergism and so possibly binds to multiple enzyme forms. Phosphono acetamide and difluoro phosphono acetamide caused little or negligible inhibition to this reaction.

Results have shown that there are compounds that bind to only C-terminal half of the large subunit and some possibly to multiple enzyme forms .Crystal structure of CPS complexed with these compounds may help differentiate between the two nucleotide binding sites and indicate the mode of binding of bicarbonate, carbamate and carbamoyl phosphate.

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VITA

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